

**REMARKS**

Applicants acknowledge receipt of the Final Rejection mailed October 1, 2009. Reconsideration and withdrawal of the outstanding rejections is respectfully requested.

Applicants have amended claim 1 to recite that the composition used in the claimed method comprises oleuropein as an “active compound.” This amendment is supported in the specification, for example, at page 16, lines 29-31.

No new matter would be added.

Claims 1-2, 4-5, 7-14, 16, 18-19, 21-23, and 25-26 are pending and under consideration. Claims 3, 6, 15, 17, 20, and 24 have been withdrawn from consideration.

**Rejection over Lockwood as evidenced by Nachman**

The Examiner has maintained the rejection of claims 1-2, 4-5, 7-14, and 25-26 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Lockwood, U.S. Patent No. 7,445,807 (“Lockwood”) as evidenced by Nachman, U.S. Patent No. 5,714,150 (“Nachman”). According to the Examiner:

“Lockwood teaches agglomerated granular protein-rich nutritional supplements, for use by specific groups of individuals. Lockwood teaches that one group to be treated include [sic] postmenopausal women, who are particularly susceptible to osteoporosis, and teaches supplements designed for women. The supplements may comprise edible plant extracts, including olive leaf extract. Olive leaf extract is known to inherently contain oleuropein; as evidence Nachman teaches a method of producing olive leaf extract known as oleuropein with valuable medicinal properties. Therefore, one skilled in the art of edible plant extracts would envisage oleuropein from the disclosure of “olive leaf extract” in Lockwood.

“Lockwood does not specifically teach that the supplement comprising olive leaf extract inhibits bone resorption

“However, it would have been obvious to a person having ordinary skill in the art at the time the invention was made to use the supplement taught by Lockwood to treat bone resorption; thus arriving at the claimed invention. One skilled in the art would be motivated to do so because Lockwood fairly teaches and suggests supplements for women, including women susceptible to osteoporosis, and Lockwood also fairly teaches and suggests the incorporation of edible plant extracts, including olive leaf extract, in said supplements. Thus one of skill in the art would be motivated to select the use of olive leaf extract in the supplement for women by routine experimentation, in order to optimize the intended use of the resulting supplement, which includes making women less susceptible to osteoporosis, thereby inhibiting bone resorption.”

(Office Action, pp. 2-3, citations omitted). Applicants respectfully traverse this rejection.

As noted in Applicants’ previous response, Lockwood is drawn to different protein-rich nutritional supplements for use by specific groups of individuals (*Abstract*), including women and older adults (*col. 1, lines 25-29*). Lockwood further teaches that nutritional needs of women, in particular of osteoporosis-susceptible postmenopausal women (*col. 1, lines 37-38*), are different from those of men (*col. 1, lines 30-31*). Lockwood describes many compounds which are supposed to help maintain healthy bones, including vitamin D, calcium, magnesium, manganese boron, and soy isoflavones. Soy isoflavones are the only edible plant extracts which Lockwood identifies as useful for treating osteoporosis or any other bone disease (*col. 4, lines 45-47*). Olive leaves are only mentioned only once in Lockwood, as part of a long list of plants suitable for use in Lockwood’s nutritional supplements (*col. 9, lines 28-41*).

Thus, Lockwood only teaches the possible treatment of osteoporosis with magnesium, calcium, manganese, phosphorous, soy isoflavones, and vitamin D; none of these compounds are structurally similar to oleuropein. Therefore, nothing in

Lockwood teaches or suggests a method of stimulating bone formation and/or inhibiting bone resorption in humans or animals comprising the administration of a composition comprising oleuropein as active compound.

With all due respect, Applicants respectfully submit that the Examiner has inadvertently engaged in hindsight analysis, relying on Applicant's disclosure to reconstruct the claimed invention. Neither Lockwood nor Nachman teach or suggest that olive leaf extracts – in particular oleuropein – have an activity towards bone metabolism. (The Examiner has essentially admitted that Nachman provides no teachings that olive leaf extracts are helpful in treating osteoporosis.) Thus, a person of skill in the art would never, based on the teachings of Lockwood and Nachman, arrive at a method of stimulating bone formation and/or inhibiting bone resorption in humans or animals comprising the administration to a subject in need thereof of a composition comprising oleuropein as active compound.

Finally, in their last response, Applicants noted that nothing in Lockwood teaches or suggests that indicate that olive leaf extracts are helpful for treating osteoporosis; nor does Nachman, which makes no mention of osteoporosis, cure this deficiency. While many plant extracts are identified in Lockwood as useful in its nutritional supplements, only one is olive leaves (the use of which is not exemplified for any purpose).

The Examiner has rejected this argument on the grounds that:

“Applicant's claims are not limited to methods wherein the active agent treating osteoporosis is oleuropein; rather the claims merely require that a composition comprising oleuropein is used to stimulate bone formation and/or inhibit bone resorption.”

(Office Action, p. 7, emphasis added). In response, Applicants reiterate that neither of the prior art documents describe a method for inhibiting bone resorption and/or

stimulating bone formation comprising the administration of oleuropein, either alone or in combination with other active compounds. Thus, Applicants submit that the presently claimed method (which recites that the oleuropein is an active agent) is clearly patentable over the cited art, even if nutritional compositions comprising olive extracts may not be.

For all of the foregoing reasons, Applicants submit that the presently claimed invention is patentable over the teachings of Lockwood in view of Nachman, and respectfully request withdrawal of this obviousness rejection.

**Rejection over Hamdi in view of Katori**

The Examiner has also rejected claims 1, 16, 18-19, and 21-23 under 35 U.S.C. § 103(a) as allegedly unpatentable over Hamdi, et al., U.S. Patent Application Publication US 2003/0004117 (“Hamdi”) in view of Katori, et al., Inflamm. Res. 49 (2000), pp. 367-392 (“Katori”). Applicants traverse this rejection.

Hamdi is drawn to methods for efficiently inhibiting angiogenesis, and for treating different inflammatory diseases associated with unwanted angiogenesis (¶¶ [0056] – [0071]). In a preferred embodiment, the administered compound is oleuropein, in particular oleuropein from the olive tree (¶ [0054]).

As previously noted by Applicants, Katori is a general review article about cyclooxygenase-2 (COX-2), which reports that that prostaglandins, whose formation is catalysed by COX-2, could be responsible for hypercalcemia and inflammatory bone loss in periodontal diseases and in rheumatoid arthritis. The main effect of prostaglandins, in particular of one thereof, could be the stimulation and inhibition of bone resorption and formation.

The Examiner claims that because Hamdi teaches methods for inhibiting angiogenesis (a condition in which COX-2 is present) with a composition comprising oleuropein, and Katori suggests that COX-2 may be present in bone resorption, it would have been obvious to devise a method of stimulating bone formation and/or inhibiting bone resorption by administering a composition comprising oleuropein.

Although the Examiner seemingly has acknowledged an obligation to avoid an hindsight-based reconstruction of Applicants' claimed invention, Applicants respectfully submit that this rejection is a classic case of forbidden hindsight-based analysis, and should be withdrawn. That the rejection is based on prohibited hindsight reconstructions is shown by the following points.

First, the Examiner has narrowly focused on COX-2 as the mechanism for both angiogenesis and bone metabolism. However, one skilled in the art would certainly understand that these biological mechanisms involve a plurality of distinct proteins and effectors other than COX-2. Thus, one of skill in the art, even if he or she would have considered the teachings of Hamdi and Kator together, would have had no reason to expect that oleuropein would have been useful in treating bone metabolism disorders; neither reference provides a connection between oleuropein and bone metabolism.

Second, neither Hamdi nor Katori describes or suggests that oleuropein is a COX-2 inhibitor. Without such a suggestion, one of skill in the art would have made the logical leap required by the Examiner's analysis.

Third, even if one of skill in the art might divine from the cited references that oleuropein could be a COX-2 inhibitor, one of skill in the art would not reasonably have expected that expect that oleuropein might stimulate bone formation and/or

inhibit bone resorption because the effect of COX-2 inhibitors on bone metabolism is completely unpredictable. This unpredictability is shown by reference to a many articles in the field, several of which are described below and copies of which are included for the Examiner's convenience:

1. Niki *et al.* (Niki *et al*, Journal of Immunology, 2007, 179;639-646) ("Niki"), mentions that *"the effect of COX-2 inhibitor on osteopenia associated with inflammatory arthritis remains controversial"* (see paragraph 2, right column, p.639) and that *"systemic administration of celecoxib (which is a well-known COX-2 inhibitor) ameliorates joint inflammation but osteopenia is rather exacerbated despite effective downregulation of PGE2, essentially contradicting past lines of evidence supporting the osteoprotective effects of COX-2 inhibitors"* (see paragraph 1, left column, p.640). More precisely, Niki shows that *"COX-2 inhibitor exerts negative effects on bone marrow density and trabecular bone quality mainly through reduced bone formation, indicating that blockade of PGE2 exacerbates osteopenia"* (see paragraph 1, right column, p.644) (Osteopenia is a condition where bone mineral density is lower than normal. It is considered by many to be a precursor to osteoporosis). Thus, Niki suggests that COX-2 inhibition may promote bone resorption.
2. Tiseo *et al* (Tiseo *et al*, Clinics, 2006 ; 61 : 223-30) ("Tiseo") relates to the effect of selective COX-2 inhibitors on bone regeneration. More precisely, Tiseo assesses the effect of rofecoxib (a selective COX-2 inhibitor) administration to Wistar rats subjected to right femur osteotomy. Tiseo shows no difference between control group and rofecoxib-administered rats group in terms of bone callus area and bone neoformation. Tiseo thus concludes that COX-2 inhibitors such as rofecoxib *"do not interfere with bone regeneration"* (see right column, p.227).
3. Endo *et al* (Endo *et al*, Journal of Physiological Anthropology and Applied Human Science, 2002, 235-238) ("Endo") describes the effect of another COX-2 specific inhibitor, etodolac, on fracture healing. Endo shows that

peritoneal administration of etodolac to Wistar rats with femoral fracture significantly prohibits maturation of callus and delays fracture healing (see Discussion part). Thus, Endo suggests that a known COX-2 inhibitor does not stimulate bone formation.

4. Finally, O'Keefe *et al* (O'Keefe *et al*, Annals of the New York Academies of Sciences, 2006, 532542) ("Endo") shows that COX-2 inhibitors delay bone allograft healing incorporation and significantly reduce bone formation, whereas PGE2 infusion (which mimics COX-2 activation) increases bone formation.

These articles demonstrate that some COX-2 inhibitors either have no effect on bone metabolism or may deeply impair bone formation. This demonstrates that the Examiner's reasoning is invalid. In other words, even if a person of skill in the art were aware from Hamdi that oleuropein might inhibit angiogenesis and gleaned (somehow) from Katori that COX-2 had some effects on bone metabolism, the person of skill in the art would also understand that COX-2 inhibitors - which are angiogenesis inhibitors according to Katori (see, p.373 of Katori) - are not necessarily able to stimulate bone formation or inhibit bone resorption. Thus, even the combination of Hamdi and Katori would not lead a person of skill in the art to the claimed method.

Accordingly, Applicant respectfully submits that the rejection over Hamdi in view of Katori be reconsidered and withdrawn.

Serial No. 10/552,723  
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**Conclusion**

In view of the foregoing, Applicants respectfully submit that the claims are in condition for allowance, and earnestly solicit prompt notice to that effect. Should the Examiner believe a personal interview would be helpful in advancing prosecution, he is invited to telephone the undersigned.

Respectfully submitted,

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# COX-2 has a Critical Role During Incorporation of Structural Bone Allografts

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**ABSTRACT:** Nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenase (COX) activity, reduced pain and are commonly used in patients with skeletal injury. In this article we will also present data to show that selective COX-2 inhibitor delays allograft healing and incorporation. In contrast, local delivery of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) enhanced bone formation at cortical bone graft junction. A 4-mm mid-diaphyseal segmental femoral defect was created and then repaired by frozen bone allograft of the same size. A 22-gauge metal pin was placed in the intramedullary cavity to stabilize the bone graft. Healing was evaluated weekly by X ray and by a semiquantitative histomorphometric analysis at 5 weeks postsurgery. Celecoxib (25 mg/kg/day) and Ketorolac (4 mg/kg/day) were administered daily for 2 weeks or 5 weeks. PGE<sub>2</sub> was infused locally at a dose of 800 nmol/kg per day via osmotic minipump for 4 weeks. Inhibition of cyclooxygenase by daily administration of the Celecoxib or Ketorolac for 5 weeks reduced new bone ingrowth by about 60% ( $P < 0.05$ ). The percentage of bony bridging in both drug-treated groups was significantly decreased at 5 weeks. Temporal administration of Celecoxib for 2 weeks also significantly reduced bone formation by 45% and withdrawal of the Celecoxib only led to slight recovery of bone formation at the graft side. In contrast to the inhibitory effects of NSAIDs, PGE<sub>2</sub> infusion at the cortical bone junction increased bone formation by about twofold. These results demonstrated that COX-2 is essential for bone allograft incorporation. Furthermore, our data support the notion that COX-2-dependent PGE<sub>2</sub> produced at the early stage of bone healing is prerequisite for efficient skeletal repair.

**KEYWORDS:** cyclooxygenase; PGE<sub>2</sub>; allografts; COX-2

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## INTRODUCTION

Nonsteroidal anti-inflammatory drugs, also known as NSAIDs, are the most commonly used pain-relieving medications in our society. In 1971, Sir John Vane demonstrated for the first time that the mechanism of action of aspirin and other NSAIDs is via inhibition of cyclooxygenase (COX), which catalyzes the formation of prostaglandins and thromboxanes from arachidonic acid (AA).<sup>1,2</sup> It is now known that at least two isoforms of COX exist, the inducible isoform COX-2 and the constitutive isoform COX-1. While NSAIDs have different selectivity toward these isoforms, inhibition of COX-2 is considered as the basis for the anti-inflammatory and analgesic effects of NSAIDs.<sup>3,4</sup> While selective COX-2 inhibitors have reduced gastrointestinal complications, recent studies have revealed that suppression of COX-2 may perturb normal renal and cardiovascular functions.<sup>5-7</sup>

Several studies show that COX activity is involved in normal bone metabolism and suggest that NSAIDs have a negative impact on bone repair. Ibuprofen, Indomethacin, Ketorolac, Celecoxib, and Rofecoxib have been shown to delay fracture healing in animal models.<sup>8-13</sup> Bone repair in animal models of spine fusion is also impaired by NSAIDs.<sup>14,15</sup> Definitive data in humans are not available since prospective, placebo-controlled clinical trials with bone healing as a primary endpoint have not been performed. However, retrospective studies have suggested that treatment with NSAIDs impairs spinal fusion in humans.<sup>16,17</sup> Retrospective studies of fracture repair in humans have shown mixed results, with some studies showing inhibition and others demonstrating no effect.<sup>18,19</sup> Consistent with an inhibitory effect on bone repair, Indomethacin and Ketorolac have been advocated for preventing heterotopic ossification following joint replacement.<sup>20-23</sup>

The most compelling data implicating COX activity during bone repair comes from genetic models that demonstrate a critical role for COX-2. Simon *et al.*<sup>24</sup> demonstrated that COX-2 *-/-* mice had impaired fracture healing whereas COX-1 knockout mice had a normal rate of repair.<sup>13</sup> Work in our laboratory also showed diminished bone repair in COX-2 *-/-* mice following fracture. Although cartilage maturation occurred in COX-2 *-/-* mice as evidenced by the expressions of *type X collagen* and *MMP-13* genes, completion of endochondral bone formation with the elimination of cartilage was impaired. Moreover, osteoblast differentiation from mesenchymal precursors was markedly impaired, with decreased intramembranous bone formation and reduced osteocalcin gene expression observed in fractures as well as on the calvarial surface following FGF-1 injection. Cell culture studies confirmed the decreased bone formation, since the number of bone nodules was reduced in COX-2 *-/-* mice. However, the decrease in bone nodule formation in COX-2 *-/-* mice is reversed by the addition of exogenous PGE<sub>2</sub>.

These later data suggest that mesenchymal cells remain receptive to PGE<sub>2</sub> and bone formation occurs normally once COX-2/PGE<sub>2</sub> signals are available.

Several studies using pharmacological inhibition of COX-2 show that after an initial delay, fracture healing progresses and approaches normal rates following either brief or continuous treatments with NSAIDs/COX-2 specific inhibitors.<sup>25</sup> However, animal fracture studies use models where healing is extremely reliable. In contrast, human fractures are heterogeneous and while some fractures, such as the distal radius, essentially always heal, other fractures, such as tibia fractures and spinal fusion have elevated rates of nonunion.<sup>26</sup> Moreover, it has been shown that comorbidity, including various diseases and the associated degree of bone and soft tissue injury impacts on fracture healing.<sup>26</sup> In the current study we used a gain and loss of function approach in order to better understand the role of COX-2 inhibition in a murine segmental femur allograft-healing model.<sup>27,28</sup> This is a more challenging model of bone repair that mimics clinical scenarios where nonunion is more likely to occur. Allografts do not provide cells to the local environment and healing has been shown to depend upon osteoinductive and angiogenic activity from the host at the cortical bone junction.<sup>29,30</sup> In our current study we examined the effects of Celecoxib at a dose of 25 mg/kg and Ketorolac at a dose of 4 mg/kg on bone formation in this model. Furthermore, we infused PGE2 locally at the cortical bone junction via osmotic minipumps and demonstrated that constitutive administration of PGE2 increased allograft healing via increasing host bone formation at the allograft junction.

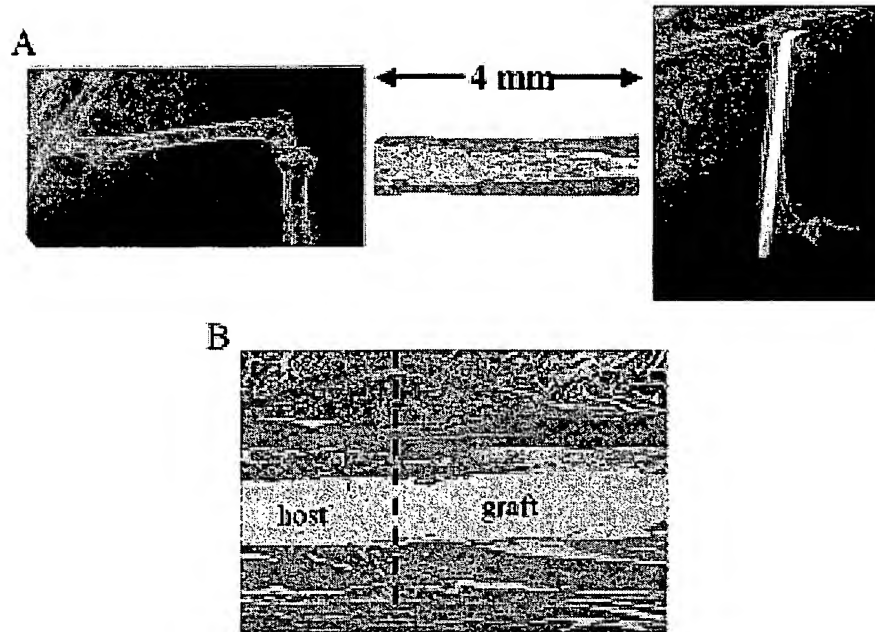
## MATERIALS AND METHODS

### *Murine Segmental Femoral Graft Model*

Ten-week-old C57BL/6 mice were anesthetized, a 7- to 8-mm long incision was made, and the femur was exposed by blunt dissection. A 4-mm mid-diaphyseal segment was removed by osteotomizing the bone using angled wire scissors. Bone grafts were obtained from the femoral shaft of a different strain of mice: 129 (allografts). These 4-mm allografts were sterilized with 70% ethanol and then fresh frozen at  $-70^{\circ}\text{C}$ . Prior to re-implantation they were thawed to room temperature and rinsed in saline to remove residual ethanol. The bone graft was secured with a 22-gauge metal pin placed through the segmental graft through the marrow cavity. The incision was closed by silk suture. Graft healing was followed radiographically using a Faxitron X-ray system (Faxitron X-ray Corporation, Wheeling, IL). Mice were sacrificed at 2, 3, 4, and 5 weeks after surgery and samples processed for histological analysis.

### *Histological and Histomorphometric Analyses*

Following sacrifice, grafted femurs were harvested, fixed in 10% neutral-buffered formalin, decalcified, and 3- $\mu\text{m}$  paraffin-embedded sections prepared



**FIGURE 1.** Schematic representation of the mouse segmental femoral bone graft model. (A) An 4-mm long allograft was used to repair the segmental defect created in a host mouse femur. The graft was stabilized via intramedullary metal pin. (B) Illustration of bone histomorphometric measurements for graft incorporation.

and stained with hematoxylin/eosin, or alcian blue/hematoxylin.<sup>28</sup> Histomorphometric analysis was carried out using Osteometrics™ software (Osteometrics, Inc., Decatur, GA) to determine the area of periosteal new bone formation. As illustrated in FIGURE 1, a line was drawn in the middle of the junction between graft and host bone to separate the new bone formation on the surface of the graft and the host bone. The measurement of periosteal bone formation on the side of the graft reflects the ingrowth of new bone into the graft, and was used to evaluate graft healing in this study.<sup>28</sup> A total 61 allografts were implanted and evaluated. Eleven specimens were excluded from the study due to infection or fracture of the graft. (See TABLE 1).

**TABLE 1.** Animals used to evaluate NSAID effects on allograft healing

	Control (5 weeks)	Celecoxib (2 weeks)	Celecoxib (5 weeks)	Ketorolac (5 weeks)
Number of animals used	16	10	20	15
Number of specimens included	16	6	17	11

### ***Drug Administration***

Celecoxib (25 mg/kg/day) was administered orally (daily for 2 or 5 weeks) and Ketorolac (4 mg/kg/day) was administered by intramuscular injection (daily for 5 weeks). Drugs therapy was initiated on the day of surgery and all animals were sacrificed at 5 weeks.

### ***PGE2 Delivery via Osmotic Minipumps***

Allograft surgery was performed and a polyvinyl catheter was fixed on the bone surface and connected to an Alzet 2002 osmotic minipump (ALZET, Cupertino, CA) implanted in the subcutaneous tissue of the back. The pumps contained PGE2 in ethanol/propylene glycol (40:60, vol/vol). Infusion was performed at a rate of 0.25  $\mu$ l/h for 4 weeks. PGE2 was infused at a dose of 800 nmol/kg/day. X rays of the femur were taken at days 0, 14, and 28. Animals were sacrificed on day 28 and femurs processed for histology.

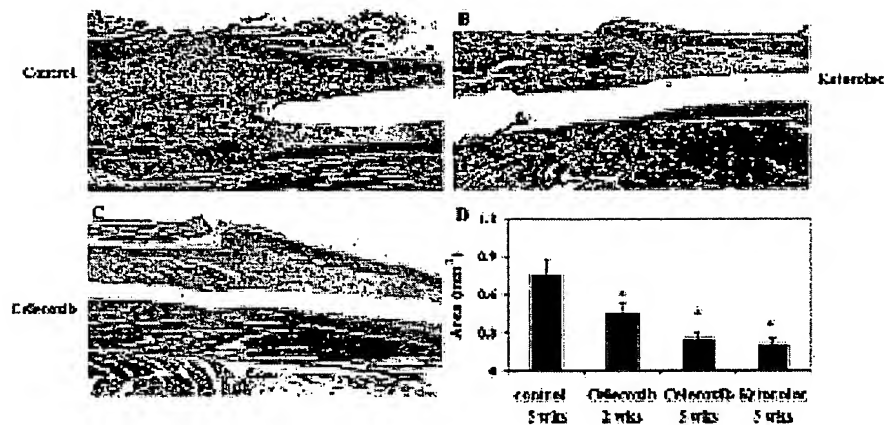
### ***Statistical Analysis***

Data are expressed as the means  $\pm$  SEM. Statistical significance was determined by one-way ANOVA. A  $P$  value  $<0.05$  was considered statistically significant.

## **RESULTS**

### ***Celecoxib or Ketorolac Treatments Reduce Bone Formation and Allograft Incorporation***

The effect of sustained and temporary COX-2 inhibition was examined on allograft healing using the selective COX-2 inhibitor Celecoxib (25 mg/kg/day) and the nonspecific NSAID, Ketorolac (4 mg/kg/day). Since allograft healing relies on the recruitment of mesenchymal stem cell from the host bed, and is limited to bone formation at the host allograft junction, we hypothesized that healing would be highly sensitive to COX-2 inhibition. Continuous COX inhibition for 5 weeks resulted in a 60% decrease in bone formation following both Celecoxib and Ketorolac treatments compared to controls ( $P < 0.05$ ) (FIG. 2). No significant differences in new bone formation were observed between the Celecoxib- and Ketorolac-treated groups. Both groups showed a lower percentage of histologic bony bridging after 5 weeks, 55% ( $n = 17$ ,  $P < 0.05$ ) and 61% ( $n = 11$ ,  $P < 0.05$ ) for Celecoxib and Ketorolac, respectively, as compared to 82% ( $n = 16$ ) in the controls. In the group that was treated with



**FIGURE 2.** Celecoxib or Ketorolac treatment inhibits bone formation on the surface of allografts. Representative histological sections show the allograft incorporation at the host graft junctions at 5 weeks postgrafting in control group (A), Ketorolac- (B), or Celecoxib-treated mice (C). Histomorphometric quantification demonstrated a significant reduction in new bone formation at the graft side in both short-term (2 weeks) and long-term (5 weeks) Celecoxib treatment groups and the group treated with ketorolac for 5 weeks (\* $P < 0.05$ ).

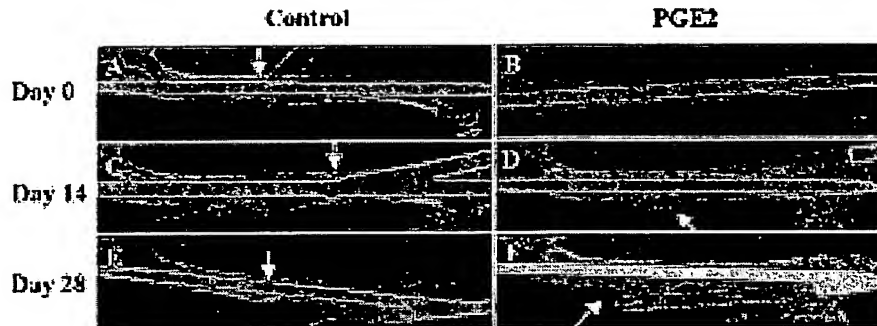
Celecoxib for only 2 weeks following surgery, a significant reduction in bone formation was also noticed, which was accompanied by a lower rate (66%) of histological bony bridging ( $n = 6$ ). While cessation of Celecoxib at 2 weeks resulted in a slight recovery in bone formation, the recovery was incomplete and a 45% reduction in bone formation remained (FIG. 2D).

#### *PGE2 Infusion Enhances Bone Formation in Allograft Model*

To examine COX gain of function, PGE2 was infused into the allograft site at a dose of 800 nmol/kg/day. Weekly radiographs demonstrated increased bone callus formation at the host/allograft junction. Callus was present at day 14 in PGE2-infused mice, while vehicle-treated mice had absent or minimal callus (FIG. 3 C,D). By day 28, callus formation was much greater in PGE2-treated mice compared to the vehicle controls (FIG. 3 E,F). Histological and histomorphometric analyses confirmed increased bone formation following PGE2 infusion (FIG. 4). Quantitative histomorphometry demonstrated about a twofold induction of bone formation on the host side and the allograft side ( $n = 3$ ).

## DISCUSSION

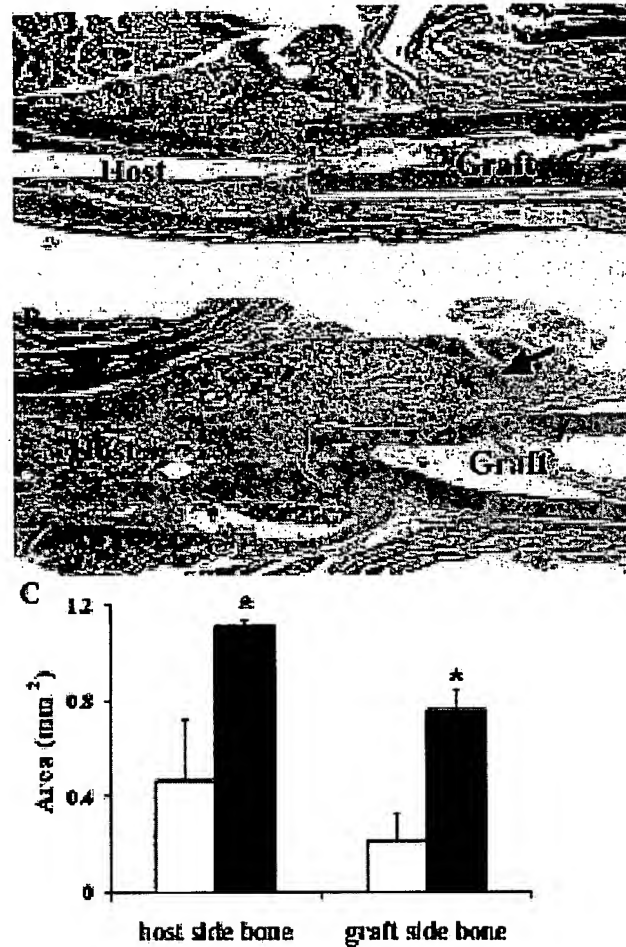
A murine allograft-healing model was used to examine the role of COX gain and loss of function in bone repair. Both Ketorolac and Celecoxib



**FIGURE 3.** Induction of bone callus formation upon *in vivo* PGE2 infusion. An osmotic minipump was inserted subcutaneously into the mouse and an 18G catheter was used to direct vehicle (A, C, E) or PGE2 (B, D, F) to the femoral grafting site. Radiographic healing was monitored by X rays at days 0 (A,B), 14 (C,D), and 28 (E,F) postsurgery. An increase in bone callus formation was observed at the site of the infusion (arrow).

markedly inhibited bone repair. Furthermore, short-term administration of Celecoxib for the initial 2 weeks continued to impair bone formation and allograft incorporation at 5 weeks. In contrast, PGE2 infusion stimulated bone formation and healing. Taken together, these results suggest that COX-2-dependent prostaglandins are essential for bone repair and PGE2 generated in the early phase of bone healing is required for efficient bone healing.

It was shown by Gerstenfeld *et al.* in a rat tibia fracture healing model that COX-2 expression is transiently increased during the first 14 days following fracture whereas COX-1 expression does not change over a period of 28 days.<sup>25</sup> The transient induction of COX-2 supports a role during the early phase of bone healing. Indeed, a study by Riew *et al.* suggests that the inhibitory effects of NSAIDs are more significant when administered earlier following spinal fusion.<sup>15</sup> Other studies also suggest that early administration of NSAIDs results in greater inhibition of bone formation.<sup>31–34</sup> However, conflicting data have been presented recently with regard to the effects of short-term inhibition of COX-2 in rat fracture healing models. Gerstenfeld and Einhorn *et al.* report a complete healing recovery in a rat fracture healing model following withdrawal of COX-2 inhibition,<sup>25</sup> whereas O'Connor *et al.* reported a significant healing impairment in a 1- to 14-day treatment regimen.<sup>35</sup> Brown *et al.*, also used a rat model and showed that fracture healing recovers to normal levels, even in the presence of continuous COX inhibition.<sup>36</sup> The current allograft model extends prior observations by examining the role of repair in a more challenging healing environment, similar to the situations commonly encountered in humans. The presence of devitalized allograft involves reduced numbers of bone-forming precursors and decreased vascularity, and therefore may be more sensitive to drug inhibition.



**FIGURE 4.** Induction of new bone formation upon *in vivo* PGE2 infusion. Representative histology showed the proximal end of bone graft junctions from vehicle (A) or PGE2-treated group (B) at 4 weeks postgrafting. Histomorphometric analyses demonstrated a significant increase in new bone formation on the surface of host and graft in allografts infused with PGE2 (C) (white bars = control, black bars = PGE2;  $n = 3$ ,  $*P < 0.05$ ).

For more than two decades it has been known that infusion of PGE2 leads to periosteal hyperostosis in infants.<sup>37</sup> Histological sections of hyperostosis revealed a thickened periosteum and fibrocartilage-like tissue covering an exuberant neocortex of closely aligned woven bone trabeculae.<sup>38</sup> The therapeutic potential of PGE2 in bone healing was not explored until very recently. Studies using both large and small animal models showed that infusion of PGE2, EP2, or EP4 agonists increase periosteum bone formation and improve cortical bone healing.<sup>39-41</sup> A single injection of EP2 agonist CP-533,536 enhances

fracture healing by increasing the size of both cartilaginous callus and bone callus. In the current study, local infusion of PGE2 enhanced allograft healing and incorporation. Consistent with prior findings PGE2 infusion stimulated host periosteal bone formation with subsequent creeping bone callus formation that extended along the host-allograft junction. The effect was opposite to the inhibitory effects of Ketorolac or COX-2-selective inhibitor Celecoxib.

In spite of compelling genetic evidence and findings in animal studies demonstrating an important role of COX-2 in bone healing, the effects of pharmacological inhibition of COX-2 in humans is unclear because of the absence of prospective randomized clinical trials.<sup>42</sup> One study examined clinical features associated with nonunion of the femoral shaft and included 32 patients with nonunion and 67 comparable patients with fracture healing.<sup>43</sup> A marked association between nonunion and the use of NSAIDs ( $P = 0.000001$ ) was observed.<sup>43</sup> In contrast, a recent retrospective human study of 80 patients undergoing instrumented posterior spine fusion using autologous bone graft showed that Rofecoxib (50 mg) administered daily for 5 consecutive days did not increase the incidence of nonunion at 1 year postoperation.<sup>44</sup> Given the conflicting data in human studies, COX-2 inhibitors should be used judiciously in following skeletal injury or reconstruction surgery, particularly in patients with a high-risk fracture or a comorbidity that could affect bone repair.

### ACKNOWLEDGMENTS

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## Cyclooxygenase-2 Inhibitor Inhibits the Fracture Healing

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**Abstract** We investigated the effects of cyclooxygenase-2 (cox-2) on fracture healing. After closed non-displaced fractures were created at the middle of both femoral shafts in 12-week-old Wister rats, a cox-2 specific inhibitor, etodolac (20 mg/day; intra-peritoneal) was administered every day for three weeks (E group). Bone union and callus formation were evaluated by weekly radiographs. Three weeks after surgery, the mechanical strength of the fractured femur was evaluated by a three-point-bending test. These results were compared with those of a vehicle control group (V group). The fracture healing score on radiographs in the E group three weeks after the surgery was  $3.3 \pm 0.9$ , and in the V group it was  $5.8 \pm 1.5$ , indicating that fracture healing was significantly poorer in the E than the V group ( $p < 0.05$ ). From the three point bending test, the ultimate strength and stiffness of etodolac-treated fractured femurs were shown to be significantly lower than those in vehicle control group ( $p < 0.05$ ). Mechanically, femurs of etodolac treated rats were weaker than those of control rats. Thus, it was concluded that etodolac, a cox-2 specific inhibitor, inhibited fracture healing. *J Physiol Anthropol* 21 (5): 235–238, 2002 <http://www.jstage.jst.go.jp/en/>

**Keywords:** fracture, callus, cyclooxygenase-2 (cox-2)

### Introduction

Health is very important to lead to a good quality daily life. Fracture is a very common injury, which deteriorates human health. Thus, adequate management for fracture healing is crucial to enable the fractured patients return to the original healthy life. During the treatment for fractured patients, pain control is sometimes required. Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit both cyclooxygenase-1 (cox-1) and -2 (cox-2), are widely used to alleviate pain. Recently, NSAIDs that

specifically inhibit cox-2 have been introduced. Since a specific cox-2 inhibitor is unlikely to cause gastric ulcer and may also be useful for preventing diseases such as colon cancer (Taketo, 1998; Sawaoka et al., 1998) and Alzheimer's disease (in t' Veld et al., 2001), it is becoming the most frequently used analgesic.

Traditional NSAIDs have been reported to delay fracture healing (Altman et al., 1995; Elves et al., 1982). However, the effects of the specific cox-2 inhibitor on fracture healing are still unclear. Fracture healing is initiated by an inflammatory response expressing a variety of cytokines and growth factors (Tatsuyama et al., 2000). Cox-2 is normally produced at inflammation phases following fracture. From such a standpoint, the specific cox-2 inhibitors would be assumed to delay fracture healing. As NSAIDs are frequently used to alleviate post-surgical and post-traumatic pain, it is essential to know whether cox-2 specific inhibitors delay fracture healing.

The purpose of this study was to investigate the effects of etodolac, a cox-2 specific inhibitor, on fracture healing using a closed non-displaced femoral shaft fracture rat model.

### Materials and Methods

#### Animal

Eight female 12-week-old Wister rats, weighing 250–300 (g), were obtained from Charles River Japan Co. Ltd. (Yokohama, Japan) and used in this study. The rats were housed individually in a temperature ( $23 \pm 2^\circ\text{C}$ ) and humidity ( $55 \pm 10\%$ ) controlled room on a 12: 12 hours light-dark cycle. The animals had free access to food and water. This study conformed to the guidelines for the care and use of laboratory animals of our university.

The rats were randomly assigned to two groups; three rats received intraperitoneally administered etodolac (a cox-2 specific inhibitor; Nippon Shinyaku Co. Ltd, Tokyo, Japan) at a dose of 20 mg/kg body weight per day (E

group), while five rats were in the vehicle control group (V group).

### Fracture model

After rats were anesthetized with sodium pentobarbital (50 mg/kg body weight), skin, subcutaneous tissue, and the capsule of the knee joint were incised and the intertrochanteric space of the distal femur was exposed by the medial patellar approach. Then, a 0.8 (mm) K-wire was inserted between the condyles to the bilateral femurs from the distal end. The capsule of the knee joint, subcutaneous tissue, and skin were sutured layer to layer by 4-0 silk.

Closed non-displaced mid shaft femoral fractures were created at the bilateral sides according to the three-point bending method proposed by Bonnarens and Einhorn (1984).

### Weekly radiographic evaluation

Immediately after surgery, and at one-, two- and three-weeks following surgery, postero-anterior radiographs were taken to evaluate callus formation and bone. A radiographic scoring system for fracture healing was used for the evaluations (An et al., 1999).

Three weeks after surgery femurs were harvested from all rats, and all soft tissue and intramedurally inserted K-wires were removed. One of the fractured femurs in each rat was randomly selected and stored at  $-20^{\circ}\text{C}$  until biomechanical testing (Table 1).

### Biomechanical measurements

One day before mechanical testing, the specimen was thawed at room temperature, and then throughout the experiment it was kept moist. A three point bending test

using the method reported by Nakamura et al. (1989) was used for the evaluation. Specimens were placed on a special metal holding device with supports located at a distance of 13 (mm), and the device was connected to an actuator of the MTS system (Test star-II, MTS Inc., Minneapolis, MN, USA). The bending force was applied midway between the supports on the anterior surface at a speed of 10 mm/min until failure.

A load displacement curve was obtained from each specimen: the ultimate strength at failure and stiffness were calculated from the curve.

### Statistical analysis

All data in this study are expressed as mean  $\pm$  S.D. For the analysis of the radiographical scoring system, the Mann-Whitney U test was used, and the unpaired t-test was applied for evaluation of data from the mechanical testing. P values less than 0.05 were taken as significant.

### Results

Figure 1 shows typical radiographs with time from each group. One week after operation, mild periosteal reaction was found in the vehicle group but none were found in the etodolac group. On the third week, bone union was observed in the vehicle group, while in the etodolac group, bone union was not completed. Using the scoring system, bone healing and callus formation on the radiographs were evaluated. Figure 2 demonstrates weekly changes of the score in each group with time, with the score in the E group significantly lower than that in the V ( $p < 0.05$ ). The results indicated that bone union and callus formation were delayed by the administration of etodolac.

The data obtained from the biomechanical testing is

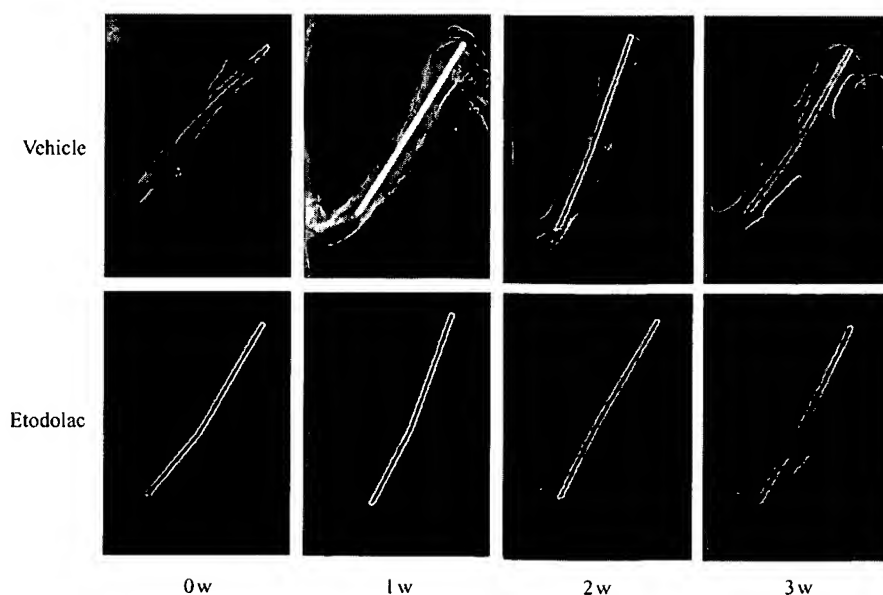


Fig. 1 Typical weekly radiographs in both groups.

shown in Figure 3. The ultimate strength was  $41.2 \pm 6.9$  and  $95.1 \pm 13.7$  (N) for E and V groups, respectively. Stiffness was  $30.4 \pm 3.9$  and  $118.6 \pm 22.5$  (N/mm) in the E and V groups, respectively. All mechanical parameters in the V group were significantly higher than those in the E ( $p < 0.05$ ).

## Discussion

In the present investigation, the effects of cox-2 specific inhibitor on fracture healing were evaluated and two major effects were clarified.

- i) Weekly radiographs demonstrated that morphologically the callus formation surrounding the fracture site, and the bone union were both poor.
- ii) Mechanical maturation, evaluated by bone strength and stiffness was inhibited.

Thereby, it was revealed that the cox-2 specific inhibitors morphologically and biomechanically prohibited maturation of callus and delayed fracture healing.

At the beginning of fracture healing, an inflammatory response is initiated. During the inflammatory period, a

variety of cytokines and growth factors such as IL-1, -6, TNF-alpha, TGF-beta and FGFs are reported to express for subsequent bone healing (Jingushi et al., 1990; Gerstenfeld et al., 2001; Einhorn et al., 1995). One of the possibilities proposed for the delayed healing is that cox-2 specific inhibitors may prohibit those growth factors that are required for fracture healing. BMP-2 is a member of the TGF-beta super-family, and is a crucial growth factor for bone formation. Koide et al. (1999) demonstrated that BMP-2 could stimulate the osteoclast-like multinucleated cell formation in presence with IL-1alpha, and that adding cox-2 specific inhibitor, NS-398, abolished the stimulation. Thus, in our model, the administration of the etodolac delayed fracture healing by prohibiting BMP-2 activity.

Zhang et al. (2002) examined the effects of cox-1 and cox-2 on bone repair using cox-1 and cox-2 knockout mice, and found that delayed endochondral bone formation in cox-2 knockout mice compared with that in cox-1 knockout mice. They also demonstrated that induced new bone formation was decreased by 60% in cox-2 -/- mice FGF-1 compared to wild type animals. Thus, in etodolac-treated rats, bone formation was involved by the inhibiting of FGFs in the reparative process of fractured bone.

No matter what the pathogenesis of delayed bone healing is, fracture healing was clearly shown to be delayed by the administration of a cox-2 specific inhibitor in the present study. As such drugs are widely used to alleviate post-surgical and post-traumatic pain, adverse effects must be considered when they are selected for use.

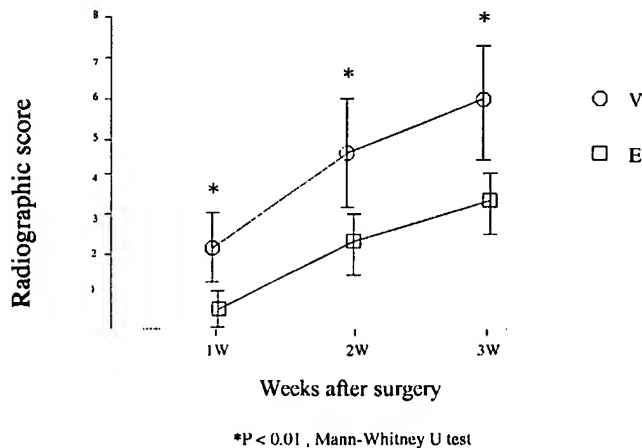


Fig. 2 Changes in radiographic scores with time.  
E, Etodolac treated rat group; V, Vehicle control group.

Table 1 Mechanically tested side of femur in each rat

Rat	1	2	3	4	5	6	7	8
E or V group	E	E	E	V	V	V	V	V
Tested side	R	R	L	R	L	L	R	R

E, Etodolac treated rat group; V, Vehicle control group; R, Right femur; L, Left femur.

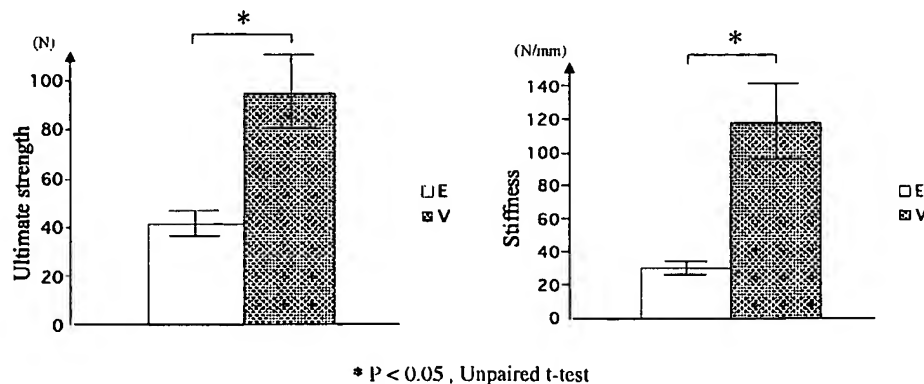


Fig. 3 Biomechanical analysis obtained by three point bending test.  
E, Etodolac treated rat group; V, Vehicle control group.

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## ORIGINAL RESEARCH

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### EXPERIMENTAL STUDY OF THE ACTION OF COX-2 SELECTIVE NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND TRADITIONAL ANTI-INFLAMMATORY DRUGS IN BONE REGENERATION

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Tiseo BC, Namur GN, de Paula EJJ, Mattar Junior R, de Oliveira CRGCM. Experimental study of the action of COX-2 selective nonsteroidal anti-inflammatory drugs and traditional anti-inflammatory drugs in bone regeneration. Clinics. 2006;61(3):223-30.

**OBJECTIVE:** The aim of this study is to compare the effects of traditional nonsteroidal anti-inflammatory drugs with nonsteroidal anti-inflammatory drugs that are selective cyclooxygenase-2 (COX-2) inhibitors in the process of bone regeneration in a rat model.

**MATERIALS AND METHODS:** Forty-four Wistar strain rats were subjected to osteotomy of the right femur and randomly divided into 3 groups according to the drug to be given (diclofenac, rofecoxib, or placebo). Each group was divided into 2 subgroups according to the time to euthanasia after the surgery. The animals of Subgroup 1 were submitted to euthanasia 2 weeks after surgery, and those of Subgroup 2, underwent euthanasia 4 weeks after surgery. Radiographic examinations and bone callus histomorphometry were analyzed.

**RESULTS:** No intergroup statistical difference was found in the bone callus area or in bone formation area 2 and 4 weeks after surgery. Intra-group analysis concerning the bone neoformation area inside the callus showed a significant difference within the diclofenac group, which presented less tissue.

**CONCLUSIONS:** Fracture consolidation in Wistar rats occurs within less than 2 weeks, and the use of nonsteroidal anti-inflammatory drugs does not significantly influence this process.

**KEYWORDS:** Anti-inflammatory agents, nonsteroidal. Bone regeneration. Fracture consolidation. Rats, Wistar. Osteotomy.

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## INTRODUCTION

Prostaglandins (PGs) are local, autacoid hormones formed by the oxygenation of arachidonic acid (AA) from cell membranes from which it is removed by phospholipase A2 (PLA2). Prostaglandins are produced by cyclooxygenase (COX), an enzyme also known as prostaglandin H2 synthase (PGHS). Cyclooxygenase is responsible for 2 reactions involving AA: the first reaction converts AA into the highly

unstable Prostaglandin G2, and the second converts Prostaglandin G2 into Prostaglandin H2. Each tissue metabolizes Prostaglandin H2 in a different manner according to the enzymes in it, generating different active metabolites of this substance. Although they are better known for their effects as mediators of inflammatory processes, Prostaglandins play an important role in other physiological processes, such as protection of the gastric mucosa, activation of the platelet system, and regulation of renal blood flow, in addition to acting as neurotransmitters.<sup>1-10</sup>

Until recently it was thought that the AA concentration, and therefore the action of phospholipase A2, was the limiting factor for the production of PGs.<sup>11</sup> It is now known that the limiting stage is the one mediated by COX.

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At first it was thought that only 1 type of COX existed. It was only in the beginning of the 1990s that studies in cells of rat ovaries<sup>12</sup> produced the first evidence for a second isoform of COX. At the same time, the conclusion was reached that the already known isoform, now called cyclooxygenase-1 (COX-1), is constitutive, meaning that it can be found regularly in tissues, while the other isoform, called cyclooxygenase-2 (COX-2), is expressed only through signaling.<sup>13</sup> Finally in 1992 the COX-2 gene was cloned.<sup>14</sup>

The fact that COX-1 is expressed constitutively while COX-2 is expressed in events such as inflammation suggests a more physiologic role for the first enzyme, while COX-2 would be more important in the production of PGs in pathological processes. Because of this, the benefit of using drugs that selectively inhibit COX-2, thus preventing frequent gastric disorders (such as ulcers), and changes in the coagulation mechanism, nephrotoxicity—all adverse reactions that prevent prolonged use of traditional nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the activity of both COX-1 and COX-2—can be clearly seen.<sup>15-19</sup>

However, it is known that COX-2 is not involved only in inflammatory processes. It is also important in a series of physiological processes such as child birth labor, when it acts by promoting mechanisms that facilitate uterine contractions.<sup>9,15</sup>

Prostaglandins play an important role in bone metabolism. Recent studies have shown that in COX-2 knockout mice compared to COX-1 knockout mice, there is also a decrease in bone resorption in response to parathyroid hormone (PTH).<sup>20</sup> In cultures of human cells, it has been shown that PTH is responsible for an increase in COX-2 expression and, consequently, of PGs in bone tissue.<sup>10</sup>

At lower concentrations,<sup>10</sup> PGs seem to have an effect on the formation of bone tissue.<sup>21,22</sup> Studies have shown that the production of lamellar bone induced by mechanical stress is mediated by COX-2.<sup>23,24</sup>

It is increasingly believed that PGs are important in bone repair, since tissue levels of PGs increase between Day 3 and Day 14 after fracture in rabbit tibias.<sup>25</sup>

Fracture consolidation is a complex process involving intramembranous bone formation and direct differentiation of mesenchymal cells into osteoblasts, as well as endochondral ossification, when the bone matrix is deposited onto cartilage.

A study that used COX-2 knockout mice showed that both types of ossification are impaired by the absence of this enzyme. The most striking point in this study was the persistence of mesenchymal cells in the site of the fracture,<sup>26</sup> pointing to an important role of PGs in the differentiation of these cells into osteoblasts. The study also

pointed to a possible role of PGs in the recruiting of mesenchymal cells as well as in remodeling of the bone scar or callus.

Based on these experimental findings and because of the use of selective COX-2 inhibitors in medical practice, it is important to study their effects of on the process of bone regeneration.

## OBJECTIVE

The objective of this study is to compare the effects of using traditional nonsteroidal anti-inflammatory drugs (NSAIDs) and NSAIDs that are selective COX-2 inhibitors in the process of bone regeneration in a rat model. We analyzed radiographic findings and histomorphometric data of the callus related with the time to regeneration of the fracture and bone neoformation area inside the callus.

## MATERIAL AND METHODS

Forty-four Wistar strain rats weighing  $341 \pm 31$  grams (mean  $\pm$  SD) were used for this study. The rats were kept in controlled conditions of temperature and lighting and with no restriction on food or water. All rats were submitted to an osteotomy of the right femur with a mechanical saw and osteotome. The rats were then randomly divided into 3 groups (1, 2, and 3) according to the drug to be given. Each group was divided into 2 subgroups according to the time to euthanasia after the surgery: Subgroup 1 2 weeks (14 days) after the surgery and Subgroup 2 4 weeks (28 days) after the surgery as follows: placebo (P), P.1 Group 2 weeks and P.2 Group 4 weeks; diclofenac (D) D.1 Group 2 weeks and D.2 Group 4 weeks; and rofecoxib (R), R.1 Group 2 weeks and R.2 Group 4 weeks.

Thirteen animals were not included in the study: 9 due to infection and 4 due to bone fixation failure (Kirschner wire migration). All groups included 5 rats each, except for the R.1 Group, which included 6 animals.

The rats in the P groups received orally 1.25 mL/kg of a solution made of 2 parts of condensed milk and 1 part of water, once daily, at the same time every day. The animals in the D groups received a similar solution of 3 mg/kg diclofenac sodium (a nonselective COX inhibitor), while the rats in the R groups received rofecoxib (a selective COX-2 inhibitor) at a dose equivalent to 1 mg/kg. These dosages were selected based in experimental and clinical studies.<sup>9,10,15-17</sup> The animals were weighed once a week for drug dose adjustment.

Fracture. All animals were anesthetized using thiopental and ketalar. The right hind limb of each rat was prepared for surgical procedure. After assembling the surgi-

cal table in a laminar air flow environment, the animal was positioned, and a 2% iodine solution was first used externally as an antiseptic.

A 2.5-cm incision was made along the femoral diaphysis. The subcutaneous and muscular planes were incised until the bone plane was reached.

The femur was exposed, a small mechanical saw was used to weaken the cortical bone layer (Figure 1), and an osteotome was used to finish the osteotomy (Figure 2).

Intramedullary bone fixation was performed using a 1.25-mm diameter Kirschner wire and an electric drill (Figures 3 and 4). The wound was closed using 4-0 nylon sutures.

The animals of Subgroups 1 (P.1, D.1, and R.1) were subjected to radiologic examination 15 days after osteotomy and 30 minutes prior to euthanasia. The animals of Subgroups 2 (P.2, D.2, and R.2) underwent a radiologic examination 2 and 4 weeks after osteotomy; the second exam was performed 30 minutes prior to euthanasia. Radiographs were taken to enable the analysis of bone union in the fracture area. Each radiograph was evaluated independently by 2 orthopedists blinded to the procedure.

After euthanasia, the femur was extracted without removing the Kirschner wire, placed in a glass container with approximately 100 mL of a 10% formol solution, and sent for histomorphometric analysis of the bone callus. A single pathologist experienced in bone tissue analysis performed the anatomical and pathological assessment. This histomorphometric assessment was blinded and measured the bone callus area and the neoformation areas inside the callus in square micrometers (Carl Zeiss AxioVision software).

The statistical analysis of the callus area and the bone neoformation area inside the callus among the 2-week subgroups (P.1 vs D.1 vs R.1) and among the 4-week subgroups (P.2 vs D.2 vs R.2) was performed using the nonparametric Kruskal-Wallis and Dunn tests. For intragroup analysis (P.1 vs P.2; D.1 vs D.2; R.1 vs R.2), the Mann-Whitney test was used. We considered  $P < 0.05$  to indicate a statistically significant difference. The total area, by individual animal, of the bone callus area and of the bone neoformation area inside the callus for each group is listed in Tables 5,6,7, and 8.

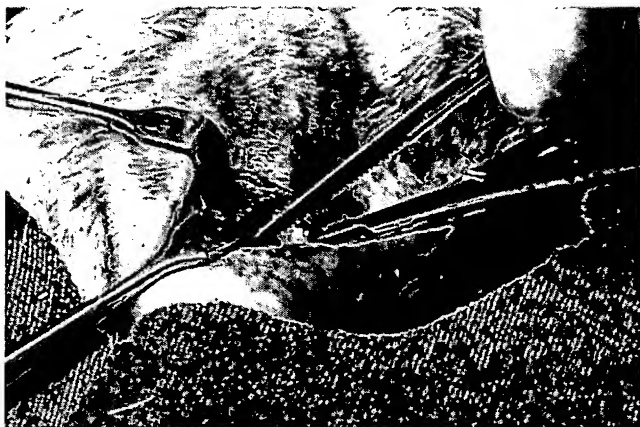


Figure 1 - Femur osteotomy with manual saw

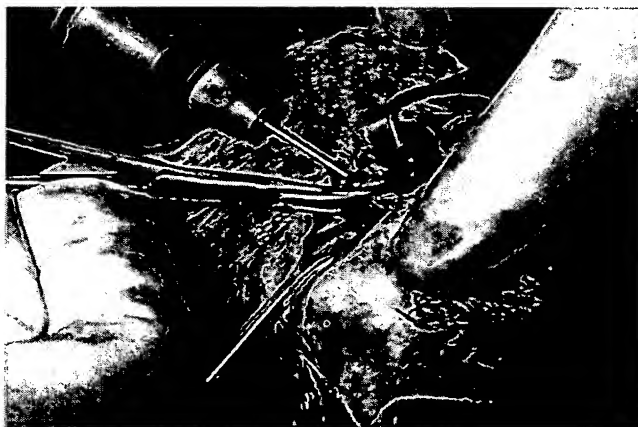


Figure 3 - Intramedullary nail fixation of the femur



Figure 2 - End of the femur osteotomy with manual saw

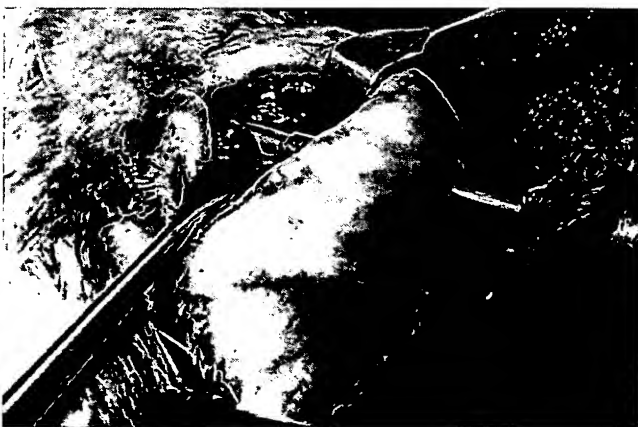


Figure 4 - Internal fixation of the femur with intramedullary nail

## RESULTS

**Radiological examination.** The orthopedists had difficulty in analyzing the X-rays. When asked to evaluate consolidation, quality of consolidation, and mechanical strength of the callus, they could only define whether consolidation was present or not. Both considered that all animals presented radiographic signs of consolidation in the examinations performed at 2 and 4 weeks after osteotomy.

**Histomorphometric examination.** In the Subgroup 1 (euthanasia at 2 weeks after osteotomy) no statistical difference was found concerning the bone callus area (Figure 5) or the bone neoformation area inside the callus  $P = 0.838$  and  $P = 0.366$ , which was confirmed by the Dunn test (Tables 1 and 3, Figure 6). In the Subgroup 2 (euthanasia at 4 weeks) there was no statistical difference in the bone callus area or in the bone neoformation area inside the callus ( $P = 0.9254$  and  $P = 0.858$ , respectively) (Tables 1 and 3).

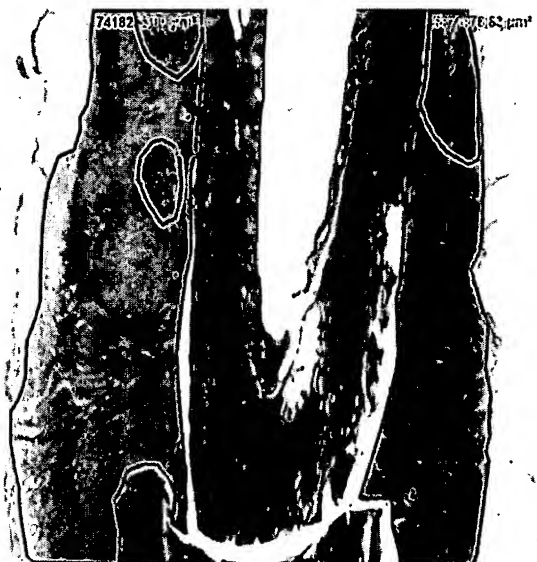
No significant intragroup difference was found regarding the amount of callus formation (Table 2). The intra-

**Table 2 - Intragroup comparison of bone callus area related to the time of sacrifice (2 vs 4 weeks after osteotomy)**

2 weeks vs 4 weeks	Bone Callus Area Data (Mann-Whitney U Test)	
	two-tailed <i>P</i> (2-tailed)	One-tailed <i>P</i> (1-tailed)
Placebo (n = 5)	1.000	0.5
Rofecoxib (n = 5)	0.7922	0.3961
Diclofenac (n = 5)	0.6905	0.34525

**Table 3 - Intergroup comparison of bone neoformation area among the groups with at 4 and 2 weeks after osteotomy**

placebo vs rofecoxib vs diclofenac	Bone Neoformation Area Data (Kruskal-Wallis and Dunn's Test)	
	(4 weeks)	(2 weeks)
global <i>p</i> =	0.858	0.366
global	$P = 0.858$	$P = 0.366$
placebo vs rofecoxib	$P > 0.05$	$P > 0.05$
placebo vs diclofenac	$P > 0.05$	$P > 0.05$
rofecoxib vs diclofenac	$P > 0.05$	$P > 0.05$



**Figure 5 - Bone callus (red)**



**Figure 6 - Bone neoformation (blue)**

**Table 1 - Intergroup comparison of bone callus area among the groups with at 4 and 2 weeks after osteotomy**

placebo vs rofecoxib vs diclofenac	Bone Callus Area Data (Kruskal-Wallis and Dunn's Test)	
	(4 weeks)	(2 weeks)
global <i>p</i> =	0.9254	0.838
global	$P = 0.9254$	$P = 0.838$
placebo vs rofecoxib	$p > 0.05$	$P > 0.05$
placebo vs rofecoxib	$P > 0.05$	$P > 0.05$
placebo vs diclofenac	$p > 0.05$	$P > 0.05$
placebo vs diclofenac	$P > 0.05$	$P > 0.05$
rofecoxib vs diclofenac	$p > 0.05$	$P > 0.05$
rofecoxib vs diclofenac	$P > 0.05$	$P > 0.05$

group analysis of the amount of bone neoformation area inside the callus showed a significant difference within the diclofenac group only (Table 4), which had less organized bone tissue.

## DISCUSSION

The radiographic examination proved to be very difficult to analyze, possibly because no comparison parameter was adopted. Since the orthopedists felt comfortable only to define presence of consolidation or absence of union of fragments, the radiograph examinations were useful for

**Table 4** - Intragroup comparison of bone neoformation area related to the time of sacrifice (2 vs 4 weeks after osteotomy)

2 weeks vs 4 weeks	Bone Neoformation Area Data (Mann-Whitney U Test)	
	two-tailed P (2-tailed)	One-tailed P (1-tailed)
Placebo	0.8413	0.42065
Rofecoxib	0.9307	0.46535
Diclofenac	0.0952	0.0476

**Table 5** - Absolute ValuesTotal area ( $\mu\text{m}^2$ ) of bone callus, by animal, area of the groups with t 4 weeks after osteotomy

placebo 4 weeks ( $\mu\text{m}^2$ )	rofecoxib 4 weeks ( $\mu\text{m}^2$ )	diclofenac 4 weeks ( $\mu\text{m}^2$ )
6641938.57	10567408.86	38890450.63
20682657.56	19344045.59	7227083.11
14712615.58	27383555.64	137919396.1
19421364.88	8211871.61	16307445
14792818.05	17969138.59	9890636.63

**Table 6** - Total area ( $\mu\text{m}^2$ )Absolute Values of bone callus, by animal, area of the groups at 2 weeks after osteotomy with 2 weeks

placebo 2 weeks ( $\mu\text{m}^2$ )	rofecoxib 2 weeks ( $\mu\text{m}^2$ )	Diclofenacdiclofenac 2 weeks ( $\mu\text{m}^2$ )
13233300.56	7197071.96	9320039.32
2446102.14	25604527.57	830794811.6
851674992.8	8652712.83	10476857.27
23812705.93	24555917.21	22604846.42
11895233.29	14904464.09	51861063.88
	14013271.87	

**Table 7** - Total area ( $\mu\text{m}^2$ ) ofAbsolute Values of bone neoformation, by animal, area of the groups with 4 weeks after osteotomy

placebo 4 weeks ( $\mu\text{m}^2$ )	rofecoxib 4 weeks ( $\mu\text{m}^2$ )	diclofenac 4 weeks ( $\mu\text{m}^2$ )
2282761.19	4857981.71	12273142.74
9033146.46	7387806.89	1519290.04
4730112.13	8147412.04	10951987.46
5769356.47	1668854.29	6712868
6447815.92	9888789.98	3212695.29

documentation purposes only. The relative stabilization obtained with the intramedullary fixation with no immobilization should have resulted in a large amount of callus, but at the end of the fourth week after osteotomy, we observed bone consolidation with no hypertrophic callus (Figure 7). Probably, a large bone callus would be seen later in this experimental model.<sup>21,24</sup>

**Table 8** - Total area ( $\mu\text{m}^2$ ) ofAbsolute Values of bone neoformation, by animal, area of the groups with at 2 weeks after osteotomy

pPlaceboo 2 weeks ( $\mu\text{m}^2$ )	rofecoxib 2 weeks ( $\mu\text{m}^2$ )	diclofenac 2 weeks ( $\mu\text{m}^2$ )
7319587.63	3866949.2	3627909.45
2030648.85	7633471.25	12852508.24
26380765.76	4268346.8	118609787.6
23812705.93	4435367.74	11641659.58
1281206.82	8305930.49	21485173.75
	8396761.08	

**Figure 7** - Radiographic image of femur consolidation

Regarding the callus area, the histomorphometric analysis showed no differences between subgroups (P.1 vs D.1 vs R.1 and P.2 vs D.2 vs R.2) at either 2 weeks or 4 weeks after osteotomy. This led us to believe that the administration of selective or nonselective non steroidal anti-inflammatory agents does not interfere with bone regeneration measured as the amount of callus formation in Wistar rats. Despite the findings of other clinical and experimental studies,<sup>21-26</sup> this model demonstrated that the administration of usually recommended doses of selective or nonselective non steroidal anti-inflammatory agents does not interfere in the ossification after bone osteotomy in the Wistar rat.

Analyzing the amount of callus formation, we did not find intragroup differences in comparing subgroups 1 and 2 (euthanasia 2 weeks and 4 weeks after osteotomy). In this model bone regeneration after bone osteotomy occurs in 2 weeks.

Concerning the comparison between the intragroup bone neoformation areas (organized bone tissue inside the bone callus), we found a significant difference within the diclofenac (D) group, since the average area of the 4-week group (subgroup 2) was smaller than that of the 2-week group (subgroup 1). It is possible that simultaneous inhibition of

COX-1 and COX-2 may explain this phenomenon, but further research should be undertaken to explain this finding.

## CONCLUSIONS

Bone consolidation in Wistar rats occurs by 2 weeks

after osteotomy, and the use of selective or nonselective nonsteroidal anti-inflammatory drugs does not interfere in the amount of callus formation between 2 and 4 weeks after the osteotomy. The inhibition of COX-1 and COX-2 may restrain the production of well-organized bone neoformation areas inside the callus.

## RESUMO

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Tiseo BC, Namur GN, de Paula EJL, Mattar Junior R, de Oliveira CRGCM. Estudo experimental da ação dos anti-inflamatórios não hormonais inibidores seletivos da ciclooxigenase 2 (COX-2) e anti-inflamatórios tradicionais na regeneração óssea. Clinics. 2006;61(3):223-30.

**OBJETIVO:** Comparar os efeitos do uso de anti-inflamatórios não-esteróides tradicionais (AINES) e AINES que são inibidores seletivos da ciclooxigenase-2 (COX-2), no processo de regeneração óssea em ratos.

**MATERIAL E MÉTODO:** Quarenta e quatro ratos da linhagem Wistar submetidos a osteotomia do fêmur direito e divididos em três grupos, conforme o medicamento que receberam (diclofenaco, rofecoxib e placebo). Cada grupo foi dividido em dois subgrupos, conforme o tempo até o sacrifício, após a cirurgia. Os animais do subgrupo 1 foram sacrificados duas semanas após a cirurgia e os do subgrupo 2, quatro semanas após a cirurgia. Foram analisados exames radiográficos e a histomorfometria do calo ósseo.

**RESULTADOS:** Não foram encontradas diferenças esta-

tísticas na área do calo ósseo 2 e 4 semanas após a cirurgia. No que se refere à área de neoformação óssea dentro do calo, observou-se diferença estatisticamente significativa apenas dentro do grupo do diclofenaco, que apresentou menos tecido.

**CONCLUSÕES:** A consolidação da fratura em ratos Wistar

ocorre dentro de 2 semanas e o uso de antiinflamatórios não-esteróides não influi de forma significativa neste processo.

**UNITERMOS:** Antiinflamatórios não esteróides. Regeneração óssea. Consolidação da fratura. Ratos Wistar. Osteotomia.

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# **Administration of Cyclooxygenase-2 Inhibitor Reduces Joint Inflammation but Exacerbates Osteopenia in IL-1{alpha} Transgenic Mice Due to GM-CSF Overproduction**

Yasuo Niki, Hironari Takaishi, Jiro Takito, Takeshi Miyamoto, Naoto Kosaki, Hideo Matsumoto, Yoshiaki Toyama and Norihiro Tada

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# Administration of Cyclooxygenase-2 Inhibitor Reduces Joint Inflammation but Exacerbates Osteopenia in IL-1 $\alpha$ Transgenic Mice Due to GM-CSF Overproduction

Yasuo Niki,<sup>1\*</sup> Hironari Takaishi,\* Jiro Takito,\* Takeshi Miyamoto,\* Naoto Kosaki,\* Hideo Matsumoto,\* Yoshiaki Toyama,\* and Norihiro Tada<sup>†</sup>

IL-1 $\alpha$  transgenic (Tg) mice exhibit chronic inflammatory arthritis and subsequent osteopenia, with IL-1-induced GM-CSF playing an important role in the pathogenesis. This study analyzed the mechanisms underlying osteopenia in Tg mice, and the therapeutic effects of the cyclooxygenase-2 inhibitor celecoxib on such osteopenia. Inhibited osteoclast formation was observed in RANKL-treated bone marrow cell (BMC) cultures from Tg mice and coculture of Tg-derived BMCs and wild-type-derived primary osteoblasts (POBs). FACS analysis indicated that this inhibition was attributable to a decreased number of osteoclast precursors within Tg-derived BMCs. Moreover, in coculture of Tg-derived POBs and either Tg- or wild-type-derived BMCs, osteoclast formation was markedly inhibited because Tg-derived POBs produced abundant GM-CSF, known as a potent inhibitor of osteoclast differentiation. Histomorphometric analysis of Tg mice revealed that both bone formation and resorption were decreased, with bone formation decreased more prominently. Interestingly, administration of celecoxib resulted in further deterioration of osteopenia where bone formation was markedly suppressed, whereas bone resorption remained unchanged. These results were explained by our *in vitro* observation that celecoxib dose-dependently and dramatically decreased osteogenesis by Tg mouse-derived POBs in culture, whereas mRNA expressions of GM-CSF and M-CSF remained unchanged. Consequently, blockade of PGE<sub>2</sub> may exert positive effects on excessively enhanced bone resorption observed in inflammatory bone disease, whereas negative effects may occur mainly through reduced bone formation, when bone resorption is constitutively down-regulated as seen in Tg mice. *The Journal of Immunology*, 2007, 179: 639–646.

Interleukin-1 is an immunomodulatory and proinflammatory cytokine that possesses a wide spectrum of biological activities, including stimulation of lymphocytes, pyrogenicity, and bone resorption (1–3). IL-1 is known as a potent activator of osteoclastic bone resorption observed in inflammatory and metabolic bone diseases such as rheumatoid arthritis, periodontitis, and postmenopausal osteoporosis (4, 5). Under these pathological situations, stimulatory actions of IL-1 in osteoclasts are mediated through both direct and indirect mechanisms. Differentiation of osteoclasts is indirectly induced by IL-1 through the induction of receptor activator of NF- $\kappa$ B ligand (RANKL)<sup>2</sup> in osteoblasts (6), whereas extended survival and pit-forming activity of osteoclasts are promoted via direct action of IL-1 (7, 8). In addition, such bone-resorbing effects of IL-1 have been documented by *in vivo* experiments, showing that s.c. injection of IL-1 in mice increases osteoclast number and bone-resorbing activity (9, 10).

Human IL-1 $\alpha$  transgenic (Tg) mice overexpressing human IL-1 $\alpha$  exhibit macrophage- and neutrophil-dominant arthritis characterized by marked synovial proliferation and progressive cartilage destruction, resembling rheumatoid arthritis with a progressive phenotype (11, 12). Of particular interest is the fact that IL-1-induced GM-CSF plays a key role in the pathogenesis of macrophage- and neutrophil-dominant inflammatory synovitis in Tg mice (11). Although IL-1 has so far been essential in osteoclastic bone resorption, great interest remains in whether bone resorption is actually enhanced in Tg mice because GM-CSF is abundantly produced in bone marrow of Tg mice, and reportedly acts as a strong inhibitor of osteoclast differentiation (13, 14). In fact, our Tg mice exhibited substantial systemic osteopenia, whereas bone resorption was rather inhibited despite genetically overproduced IL-1, contrary to our primary expectation (15). These paradoxical effects of constitutively overproduced IL-1 on bone resorption should be considered a consequence of the relative balance between RANKL as a bone resorption activator and GM-CSF as a bone resorption inhibitor, both of which are strongly induced by IL-1.

To date, numerous studies have shown that administration of cyclooxygenase (COX)-2 inhibitor reduced severity of joint inflammation in several arthritis models (16–19), but the effect of COX-2 inhibitor on osteopenia associated with inflammatory arthritis remains controversial and a subject of intense investigation. The prevention of PGE<sub>2</sub> synthesis by pharmacological or genetic ablation of COX-2 inhibits PGE<sub>2</sub>-induced RANKL expression in osteoblasts and subsequent osteoclast formation (20, 21), resulting in recovery of osteopenia. Conversely, contrasting results have been reported with selective depletion of PGE<sub>2</sub> action using a selective receptor antagonist directly abrogating the osteogenic potential of bone marrow and biasing bone metabolism toward

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<sup>2</sup> Abbreviations used in this paper: RANKL, receptor activator of NF- $\kappa$ B ligand; Tg, transgenic; POB, primary osteoblast; BMC, bone marrow cell; TRAP, tartrate-resistant acid phosphatase; COX, cyclooxygenase; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; MNC, multinuclear cell; WT, wild type.

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osteopenia (22). The net balance of bone formation vs resorption following anti-PGE<sub>2</sub> treatment may thus be changeable according to whether the dominant action of treatment is inhibition of the anabolic or catabolic actions of PGE<sub>2</sub>.

As IL-1 is reportedly a strong inducer of COX-2 and subsequent PGE<sub>2</sub> production (6, 23, 24), IL-1 $\alpha$  Tg mice should offer a useful model for the analysis of COX-2 implications in the development of inflammatory arthritis and associated osteopenia. The present study analyzed the detailed mechanisms underlying osteopenia caused by genetically overproduced IL-1 and the effects of the COX-2 inhibitor celecoxib on inflammatory arthritis and osteopenia using IL-1 $\alpha$  Tg mice. The results indicate that GM-CSF induced by genetically overproduced IL-1 $\alpha$  constitutively inhibits osteoclastogenesis in Tg mice bone marrow, both in vivo and in vitro. Under such situations, systemic administration of celecoxib ameliorates joint inflammation, but osteopenia is rather exacerbated despite effective down-regulation of PGE<sub>2</sub>, essentially contradicting past lines of evidence supporting the osteoprotective effects of COX-2 inhibitors.

## Materials and Methods

### Generation of Tg mice

The generation of human IL-1 $\alpha$  Tg mice has been previously described (11). Briefly, human IL-1 $\alpha$  cDNA was ligated into a plasmid containing CAG promoter constructed by the first intron of the chicken  $\beta$ -actin gene and a portion of the rabbit  $\beta$ -globin gene. The resulting construct was used for microinjection, and two mouse lines, Tg1705 and Tg1706, were established. The Tg1706 line was backcrossed with C3H/HeN mice for six to eight generations and used in all experiments. All procedures for animal care were approved by the animal management committees of Keio University (Tokyo, Japan).

### Reagents

Collagenase, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), and anti-MEM were purchased from Wako Biochemicals. FBS was obtained from Life Technologies. Recombinant mouse M-CSF was from R&D Systems (Minneapolis, MN), and recombinant mouse soluble RANKL was from PeproTech. PGE<sub>2</sub> was purchased from Sigma-Aldrich. Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-pyrazol-1-yl] benzenesulfonamide), which is known as a specific inhibitor of COX-2 (25), was obtained from Pfizer.

### Culture of bone marrow macrophages

Tibiae and femora were aseptically dissected from 8- to 10-wk-old male human IL-1 $\alpha$  Tg mice and C3H wild-type (WT) mice. Bone ends were removed and the bone marrow was forced out into anti-MEM containing 10% FBS. After filtration through a Cell Strainer (BD Falcon) to remove bone particles, obtained bone marrow cells (BMCs) were cultured with M-CSF (50 ng/ml) and various concentrations of RANKL (0, 10, or 50 ng/ml) in 24-well plates (Corning) at  $3.5 \times 10^5$  cells/well. After 6 days, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). The number of osteoclasts was counted as TRAP-positive multinuclear cells (MNCs) containing >3 nuclei per cell.

### Coculture of primary osteoblasts and BMCs

Calvariae were dissected from newborn Tg and WT mice. Primary osteoblasts (POBs) were obtained from calvariae by conventional enzymatic digestion using collagenase (26). POBs ( $3 \times 10^4$ ) and BMCs ( $3 \times 10^5$ ) were cocultured in anti-MEM containing 10% FBS in 24-well tissue culture plates (Corning). Cocultures were incubated with  $10^{-8}$  M 1,25(OH)<sub>2</sub>D<sub>3</sub> and  $10^{-6}$  M PGE<sub>2</sub> for 8 days. Cocultures were then fixed with 10% formalin (Wako Pure Chemicals) and stained with TRAP. The number of TRAP-positive MNCs containing >3 nuclei per cell was counted as osteoclasts.

### Flow cytometry

The cell-staining procedure for flow cytometry was performed as previously described (27). The mAbs used in immunofluorescence staining were anti-F4/80 Ab (BM8; eBioscience), anti-c-Kit Ab (2B8; eBioscience), anti-Mac-1 Ab (M1/70; BD Pharmingen), and anti-c-Fms Ab (AFS98; eBioscience) and were labeled with PE, allophycocyanin, FITC, and

PE, respectively. After 30-min incubation with fluorescein-labeled mAb, cells were washed twice with 5% FBS/PBS (washing buffer) and applied to flow cytometric analysis using FACSCalibur (BD Immunocytometry Systems).

### Administration of celecoxib

Four-week-old Tg and WT mice were divided into five groups of four mice each, and 10 or 100 mg/kg of celecoxib diluted in 0.5% methylcellulose was orally administered to each mouse every 2 days for 2 mo. All mice were killed at 8 wk after commencement of oral administration. At the end of the experiment, peripheral blood was collected by cardiac puncture for determination of PGE<sub>2</sub> levels in serum.

### Bone marrow density measurement

Femora were extracted from Tg mice and WT controls, and were fixed with 70% ethanol after the removal of soft tissues. Microcomputed tomography (CT) analysis of the femoral metaphysis and diaphysis was performed with a composite x-ray analyzing system (NS-ELEX). Femoral bone marrow density was measured by dual-energy x-ray absorptiometry using a DCS-600R analyzer (Aloka).

### Histomorphometric measurement

Tibiae were removed from Tg mice and WT controls, fixed in 70% ethanol and embedded in glycol methacrylate without decalcification. Serial sections (3- $\mu$ m thick) were cut using a microtome (model 2050; Reichert Jung). Alkaline phosphatase and TRAP staining was performed to identify the cellular components. For double-fluorescence labeling studies, all mice were s.c. injected with calcein (16 mg/kg body weight) at 11 and 4 days before sacrifice. Histomorphometric analysis of trabecular bone was performed in an area 1.8 mm long from 0.1 mm below the growth plate at the proximal tibial metaphysis using a semiautomated system (Osteoplan II; Carl Zeiss) with measurements made at a magnification of  $\times 400$ . Nomenclature, symbols and units are those recommended by the American Society for Bone Mineral Research Histomorphometry Nomenclature Committee.

### Clinical and histological assessment of arthritis

Clinical symptoms of arthritis in all four limbs were macroscopically evaluated according to a visual scoring system as reported previously (12). Arthritic joints were graded on a scale of 0–4. Scores for each mouse comprised the sum of scores for all four limbs, with a maximum score of 16. Clinical severity was also determined by quantifying changes in paw volume using plethysmometry (model 7140; Ugo Basile). In histological evaluations, paws and knees were dissected and fixed in formalin. Sagittal sections (6  $\mu$ m) were prepared and stained using H&E. As previously described (12), synovial infiltration and cartilage destruction were scored on four semiserial sections of each specimen at 10-section intervals. Neutrophil infiltration and cartilage destruction were graded on a scale of 0–3 each. Scores for each mouse comprised the sum of scores for knee and ankle joints of a hind limb, with a maximum score of 12.

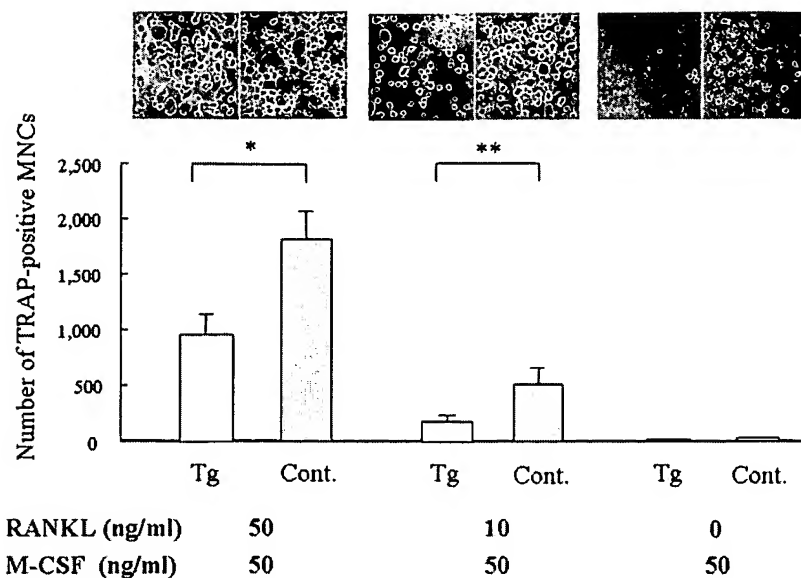
### Quantification of cytokines and PGE<sub>2</sub> production

Levels of mouse GM-CSF, human IL-1 $\alpha$ , and mouse soluble RANKL secreted into culture medium were assayed by quantitative sandwich ELISA (R&D Systems) according to the instructions of the manufacturer. Levels of PGE<sub>2</sub> in serum were also determined using a PGE<sub>2</sub> assay kit (R&D Systems). Values were determined in triplicate. Sensitivity of the assay (minimum detectable dose) is <5.0 pg/ml for GM-CSF, 4.8 pg/ml for RANKL, <1.0 pg/ml for human IL-1 $\alpha$ , and 10.1 pg/ml for PGE<sub>2</sub>.

### Analysis of in vitro osteogenesis

For induction of osteogenesis, POBs were inoculated at a density of  $5 \times 10^4$  cells/well in a 24-well plate, and cultured in medium supplemented with 50  $\mu$ g/ml ascorbic acid, 10 mM  $\beta$ -glycerophosphate, 100 nM dexamethasone (Sigma-Aldrich), and 200 ng/ml recombinant human bone morphogenetic protein BMP2 (R&D Systems). Alkaline phosphatase, Alizarin red, and von Kossa stainings were performed 7 days after confluence. For alkaline phosphatase staining, cells were fixed in 70% ethanol and stained for 10 min with a solution containing 0.01% naphthol AS-MX phosphate disodium salt (Sigma-Aldrich), 1% *N,N*-dimethylformamide (Wako Pure Chemicals), and 0.06% fast blue BB (Sigma-Aldrich). For Alizarin red staining, cells were fixed in 10% formalin/PBS and stained for 10 min with 2% Alizarin red (pH 4.0) (Sigma-Aldrich) solution. For von Kossa staining, cells were fixed with 100% ethanol and stained for 10 min with 5% silver nitrate solution (Wako Pure Chemicals) under UV light, then

**FIGURE 1.** TRAP-positive MNC formation in bone marrow cultures from Tg mice (Tg) and WT controls (Cont.). BMCs were obtained from Tg and control mice and cultured for 6 days in the presence of the indicated concentrations of RANKL and M-CSF. Cells were fixed and stained for TRAP. TRAP-positive MNCs containing three or more nuclei were counted as osteoclasts, and micrographs (top) correspond to data shown in histogram. Results are expressed as mean  $\pm$  SD of quadruplicate wells from three different experiments. \*,  $p < 0.01$ ; \*\*,  $p < 0.05$  between Tg and control mice.



incubated for 5 min with 5% sodium thiosulfate solution (Wako Pure Chemicals).

#### RT-PCR analysis

POBs cultured in anti-MEM containing 10% FBS were treated with  $10^{-8}$  M  $1,25(\text{OH})_2\text{D}_3$  and  $10^{-6}$  M  $\text{PGE}_2$  when cells reached confluence. In some cultures, various concentrations of celecoxib were added. After 3 days of culture, total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies). Reverse transcription of cDNA was performed using SuperScript III (Invitrogen Life Technologies). PCR was performed with a Titanium PCR kit (Clontech Laboratories), and  $\beta$ -actin was used as an internal control. The number of PCR cycles was selected in the linear amplification phase for each primer. For calculation of relative mRNA expression, the mRNA level for each cytokine was normalized with that of corresponding  $\beta$ -actin using NIH Image 1.6 software. Primers used for RT-PCR were as follows: human IL-1 $\alpha$  (sense) 5'-TTAC AGCACACAGGCCACCTT-3', (antisense) 5'-CGTGACGTTGCAGATCAGTTG-3'; mouse alkaline phosphatase (sense) 5'-CCAAGACGT ACAACACCAACGC-3', (antisense) 5'-AAATGCTGATGAGGTCCA GGC-3'; mouse RANKL (sense) 5'-TATGATGGAAGGCTCATGGT-3', (antisense) 5'-TGTCCTGAACCTTGAAAGCC-3'; mouse GM-CSF (sense) 5'-GGAAGCATGTAGAGGCCATCA-3', (antisense) 5'-TCCG CATAGGTGGTAACCTTG-3'; mouse M-CSF (sense) 5'-CTCTGGC TGGCTTGGCTTGG-3' and (antisense) 5'-GCAGAAGGATGAGGTT GTG-3'; and mouse  $\beta$ -actin (sense) 5'-GGCCCAGAGCAAGAGAGG TATCC-3', (antisense) 5'-ACGCACGATTTCCCTCTCAGC-3'.

#### Statistical analysis

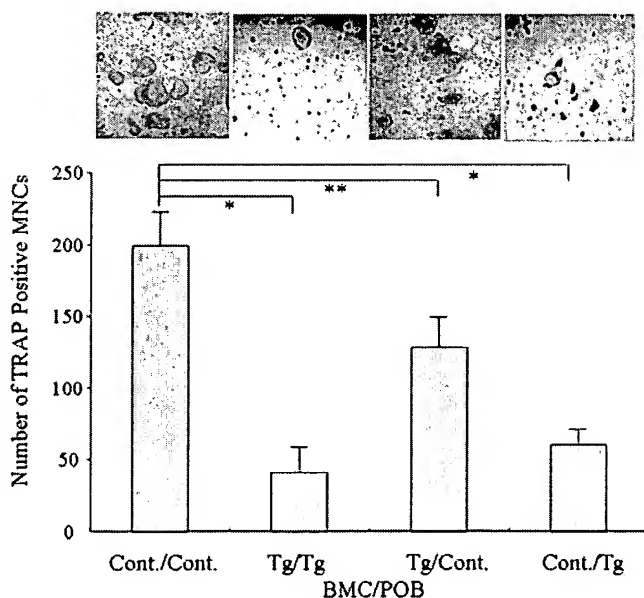
Results are basically expressed as mean  $\pm$  SD. Statistical differences between groups were determined using one-way ANOVA followed by post hoc testing using Bonferroni's method (StatView-J 5.0 statistical software; SAS Institute). Values of  $p < 0.05$  were considered statistically significant.

## Results

### Inhibition of osteoclastogenesis in Tg mice

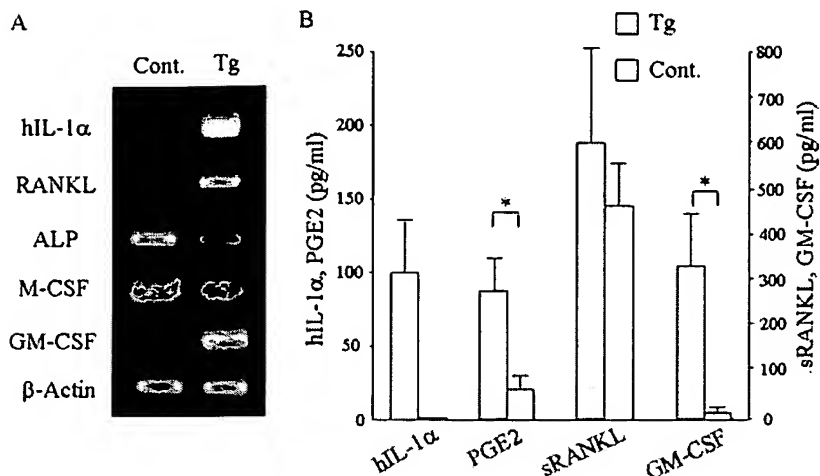
Previous histomorphometric analyses have revealed that in our Tg mice, human IL-1 $\alpha$  overproduction results in inhibited bone formation and resorption compared with WT mice, but inhibition of bone formation exceeds that of bone resorption, leading to systemic osteopenia (15). We first examined in vitro osteoclast formation induced by RANKL in cultures of bone marrow macrophages derived from Tg mice. TRAP-positive MNCs formation induced by RANKL plus M-CSF was significantly inhibited in Tg mouse-derived BMCs, as compared with control-derived BMCs, although RANKL concentration-dependent osteoclastogenesis was commonly observed in both Tg and control mice (Fig. 1).

As a next experiment, osteoclast formation was examined in the coculture of BMCs and POBs. Tg- or control-derived BMCs were cocultured with POBs derived from calvariae of Tg or control mice. Under stimulation with  $1,25(\text{OH})_2\text{D}_3$ , formation of TRAP-positive MNCs was prominent in coculture of control POBs/BMCs, but such formation was reduced by 78%, 66%, and 35% in cocultures of Tg BMCs/POBs, control BMCs/POBs, and Tg BMCs/control POBs, respectively, compared with control POBs/BMCs (Fig. 2). Consequently, osteoclastogenesis may be inhibited when either POBs or BMCs were derived from Tg mice. In addition, POBs appeared to contribute to such inhibition of osteoclastogenesis more closely than BMCs.



**FIGURE 2.** TRAP-positive MNC formation in cocultures of osteoblasts and BMCs from Tg mice and WT controls. Calvaria-derived POBs and BMCs prepared from Tg mice (Tg) and control mice (Cont.) were cocultured for 8 days in the presence of  $1,25(\text{OH})_2\text{D}_3$  and  $\text{PGE}_2$ . Cells were fixed and stained for TRAP. TRAP-positive MNCs containing three or more nuclei were counted as osteoclasts, and micrographs (top) corresponding to data in histogram are shown. Results are expressed as mean  $\pm$  SD of quadruplicate wells from three different experiments. \*,  $p < 0.001$ ; \*\*,  $p < 0.05$ , compared with coculture of control BMCs and control POBs.

**FIGURE 3.** Up-regulated production of RANKL, PGE<sub>2</sub>, and GM-CSF in Tg mouse-derived POBs. **A**, mRNA expression of transgene-derived human IL-1 $\alpha$  (hIL-1 $\alpha$ ), RANKL, alkaline phosphatase (ALP), M-CSF, and GM-CSF in POBs prepared from Tg and control mouse calvariae. **B**, Levels of human IL-1 $\alpha$ , PGE<sub>2</sub>, soluble RANKL, and GM-CSF in conditioned medium of POBs were determined using specific ELISA. Results are expressed as mean  $\pm$  SD of quadruplicate wells from three different mice. \*,  $p < 0.001$  between Tg and control mice.



#### Accelerated PGE<sub>2</sub>, RANKL, and GM-CSF production in Tg mouse-derived POBs

Because inhibition of osteoclastogenesis was closely related to Tg mouse-derived POBs, production of bone metabolism-related molecules by POBs was analyzed at the mRNA and protein levels using RT-PCR and specific ELISA (Fig. 3). As expected, Tg mouse-derived POBs displayed high levels of RANKL and GM-CSF mRNA in addition to transgene-derived human IL-1 $\alpha$ . Furthermore, significantly high levels of PGE<sub>2</sub> and GM-CSF secreted into culture medium were observed in Tg mice. Interestingly, GM-CSF was prominently induced at both mRNA and protein levels in Tg mice as compared with control mice, whereas levels of M-CSF mRNA were equivalent between Tg and control mice. Conversely, levels of alkaline phosphatase mRNA were diminished in Tg mice compared with control mice.

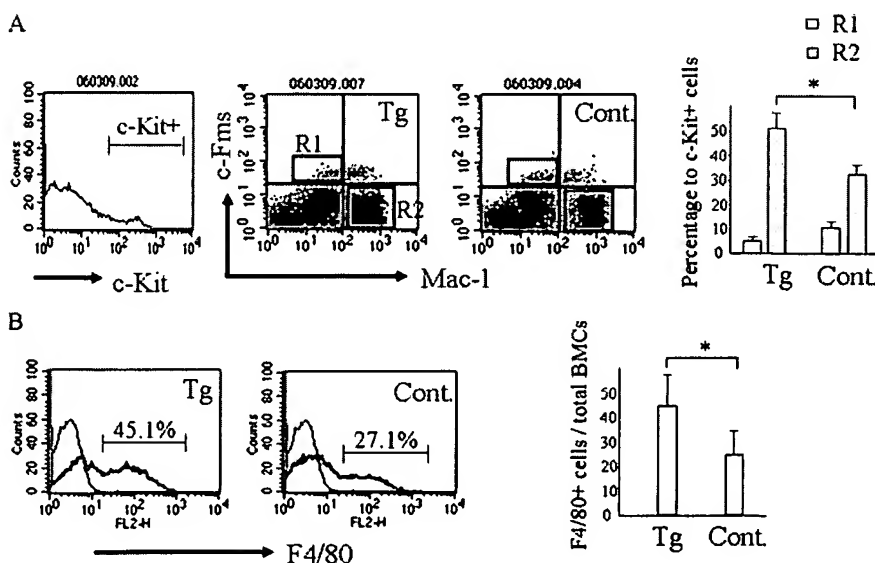
#### FACS analysis of monocyte/macrophage progenitor cells in Tg mice

To characterize monocyte/macrophage progenitor cells in bone marrow, cellular phenotypes of bone marrow mononuclear cells were analyzed using FACS. After gating on c-Kit<sup>+</sup> cells, which involve a majority of hemopoietic progenitor cells in bone marrow, cells were subdivided according to Mac-1 (CD11b) and c-Fms (Fig. 4A). In Tg mice bone marrow, c-Fms<sup>+</sup>Mac-1<sup>low</sup> (Fig. 4, R1) and c-Fms<sup>+</sup>Mac-1<sup>high</sup> (Fig. 4, R2) cells accounted for ~50% and

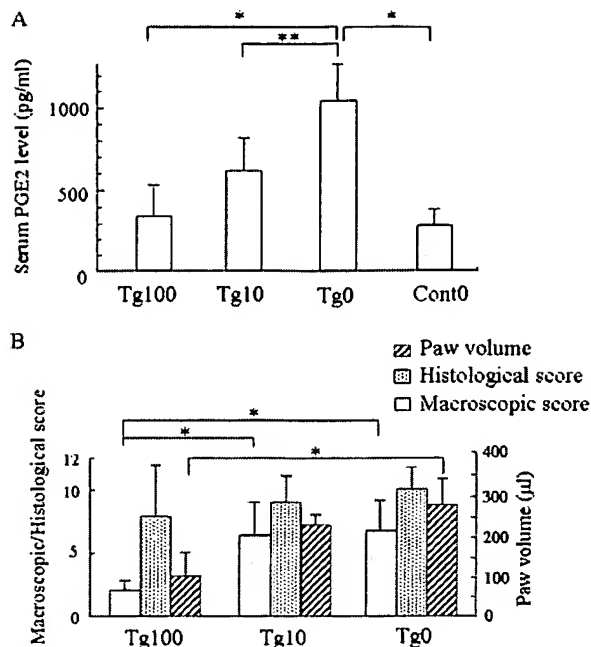
4% of c-Kit<sup>+</sup> cells, respectively. These data indicate that bone marrow of Tg mice includes a significantly larger number of c-Fms<sup>+</sup>Mac-1<sup>high</sup> cells compared with WT control. In contrast, the number of c-Fms<sup>+</sup>Mac-1<sup>low</sup> cells was lower in Tg mice than in WT controls. According to previous reports, c-Fms<sup>+</sup>Mac-1<sup>low</sup> cells comprise a large number of osteoclast precursor cells, whereas c-Fms<sup>+</sup>Mac-1<sup>high</sup> cells mainly comprise cells committed to becoming mature macrophages or dendritic cells (27, 28), suggesting that Tg bone marrow progenitor cells tend to be committed to become macrophages or dendritic cells rather than osteoclasts. Furthermore, the number of F4/80<sup>+</sup> cells, known as a highly differentiated phenotype of a macrophage lineage, was significantly higher in Tg mice than in controls (Fig. 4B), reflecting the predominance of c-Fms<sup>+</sup>Mac-1<sup>high</sup> cells in c-Kit<sup>+</sup> hemopoietic cells in Tg mice bone marrow.

#### Anti-inflammatory effects of COX-2 inhibitor in IL-1 $\alpha$ Tg mice

Because IL-1 is widely accepted as a strong inducer of COX-2 (6, 23, 24) and COX-2-induced PGE<sub>2</sub> has been implicated in several inflammatory arthritis models in mice (16–19), the anti-inflammatory effects of the COX-2 inhibitor celecoxib were examined in Tg mice. After administration of celecoxib for 2 mo, serum levels of PGE<sub>2</sub> constitutively increased in Tg mice were efficiently decreased with increasing dosages of celecoxib. In particular, PGE<sub>2</sub> levels in Tg mice declined to levels almost



**FIGURE 4.** FACS analysis of phenotypic characteristics of BMCs derived from Tg mice (Tg) and WT controls (Cont.). **A**, Expression of c-Fms and Mac-1 (CD11b) on BMCs was analyzed. After gating on c-Kit<sup>+</sup> cells, the cells were further subdivided into the following two fractions based on expression of c-Fms and Mac-1: c-Fms<sup>+</sup>Mac-1<sup>low</sup> (R1) and c-Fms<sup>+</sup>Mac-1<sup>high</sup> (R2). Representative data from four different mice are shown. Percentages of R1 and R2 fractions to c-Kit<sup>+</sup> cells are shown (far right). **B**, Expression of F4/80 on BMCs was analyzed. Percentages of F4/80<sup>+</sup> cells in Tg and control mice are shown (far right). Representative data from four different mice are shown. Results are expressed as mean  $\pm$  SD. \*,  $p < 0.05$  comparing Tg and control mice.

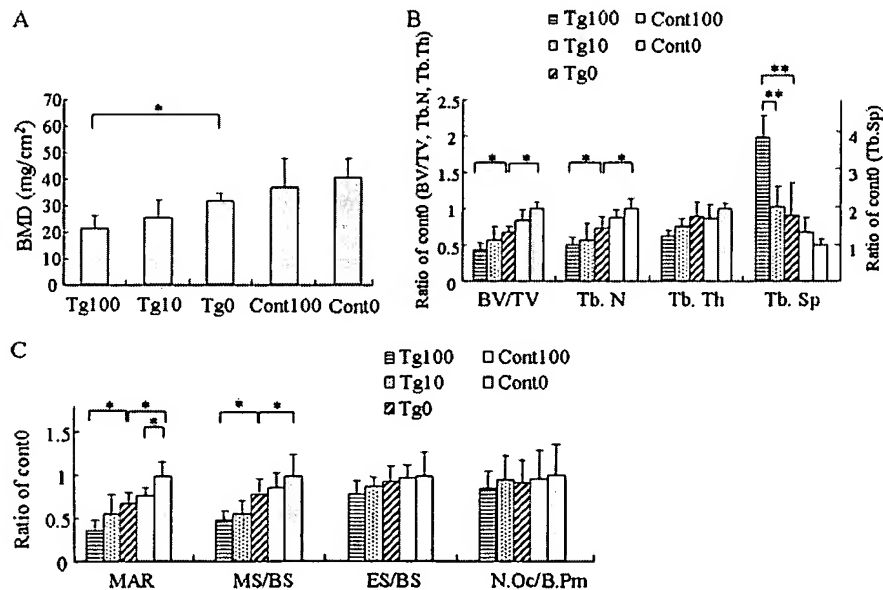


**FIGURE 5.** Anti-inflammatory effects of the COX-2 inhibitor celecoxib on arthritis in Tg mice. Tg mice (Tg) and WT controls (Cont) were orally administered as follows: Tg mice, vehicle only (Tg0); 10 mg/kg celecoxib (Tg10); 100 mg/kg celecoxib (Tg100) and WT mice, vehicle only (Cont0); 100 mg/kg celecoxib (Cont100). **A**, After 2-mo administration, serum levels of PGE<sub>2</sub> were measured in celecoxib- and vehicle-treated Tg mice and vehicle-treated WT controls. **B**, Antiarthritic effects of celecoxib were evaluated both clinically and histologically using the scoring system. Simultaneously, volumes of hind paws were measured. Each value for paw volume represents the mean of both hind paws. \*,  $p < 0.001$ ; \*\*,  $p < 0.05$  between the two groups.

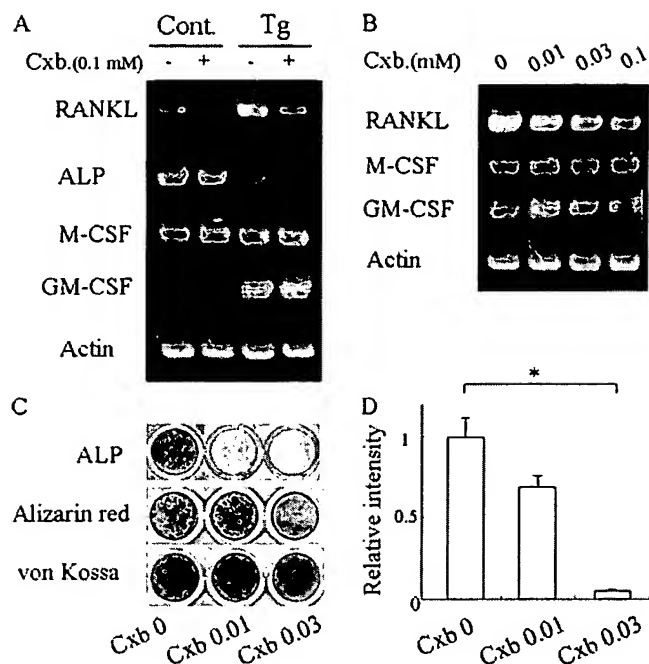
equivalent to levels in WT controls, when 100 mg/kg/day celecoxib was administered (Fig. 5A). Degree of paw swelling and macroscopic scores also decreased in a dose-dependent manner in parallel with reduced PGE<sub>2</sub> levels in celecoxib-treated mice. Histological severity of arthritis after celecoxib treatment tended to display a similar but nonsignificant decrease (Fig. 5B).

#### Effects of COX-2 inhibitor on osteopenia in Tg mice

IL-1 $\alpha$  overproduction in Tg mice resulted in reduced bone marrow density in the whole tibia, compared with WT control. Loss of bone marrow density was more prominent in the proximal and distal portions than in the central portion (data not shown). Tg mice administered celecoxib at 100 mg/kg/day displayed significant reductions in bone marrow density compared with Tg mice treated using vehicle alone (Fig. 6A). Likewise, WT control treated with 100 mg/kg/day tended to display a nonsignificant reduction in bone marrow density compared with control treated using vehicle alone. Effects of celecoxib were further analyzed using histomorphometry. Osteopenia in Tg mice has been attributed to decreased bone formation rather than increased bone resorption (15), and identical results were obtained in the present study. Various parameters for trabecular bone structure indicated trabecular bone loss in vehicle-treated Tg mice as compared with vehicle-treated WT control (Fig. 6B). Parameters for bone formation, including mineral apposition rate and mineralizing surface/bone surface ratio, were inhibited in vehicle-treated Tg mice, whereas indices of bone resorption, including eroded surface/bone surface ratio and osteoclast number/bone perimeter ratio, did not differ between vehicle-treated Tg mice and WT controls (Fig. 6C). Interestingly, administration of celecoxib resulted in loss of trabecular bone, supporting the bone marrow density data (Fig. 6B). According to the results of histomorphometry, bone formation parameters such as mineral apposition rate and mineralizing surface/bone surface ratio were further suppressed after celecoxib administration,



**FIGURE 6.** Effects of celecoxib on bone marrow density and histomorphometry. **A**, Bone marrow density (BMD) of whole femora was measured in celecoxib- and vehicle-treated Tg mice and WT controls using dual-energy x-ray absorptiometry. **B**, Histomorphometric analysis of trabecular bone in tibiae obtained from celecoxib-treated Tg mice and WT controls. Some Tg and WT mice were treated with vehicle alone. Trabecular bone samples were measured in an area 1.8 mm long from 0.1 mm below the growth plate at the proximal tibial metaphysis. Several parameters for trabecular bone (Tb) structure, including bone volume fraction (BV/TV, bone volume/total tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; and Tb.Sp, trabecular separation) are shown. Results are expressed as a ratio to WT controls treated with vehicle alone and are the mean  $\pm$  SD for four different mice. **C**, Parameters for bone formation ratios, including mineral apposition rate (MAR) and mineralizing surface/bone surface (MS/BS), and for resorption, such as eroded surface/bone surface (ES/BS) and osteoclast number/bone perimeter (N.Oc/B.Pm) are shown. Values are expressed as the ratio to WT controls treated with vehicle alone, and represent mean  $\pm$  SD for four different mice. \*,  $p < 0.05$ ; \*\*,  $p < 0.001$  between the two groups.



**FIGURE 7.** Effects of celecoxib on mRNA expression of bone metabolism-related agents in POBs. Tg or WT control (Cont.) mouse-derived calvarial POBs were cultured with or without indicated concentrations of celecoxib for 3 days. **A**, RT-PCR analysis of mRNA expression levels in POBs. Ethidium bromide-stained RT-PCR products are shown. **B**, Dose-dependent effects of celecoxib on each mRNA expression. **C**, After 7-day culture, the effects of celecoxib on osteogenesis in 24-well plates were assessed using alkaline phosphatase (ALP), Alizarin red, and von Kossa stainings. **D**, Relative intensity of Alizarin red staining was expressed as a ratio to control POBs not cultured without celecoxib (Cxb0). Results are mean  $\pm$  SD of triplicate wells from three different mice. \*,  $p < 0.001$ .

whereas bone resorption parameters such as eroded surface/bone surface and osteoclast number/bone perimeter ratios remained largely unchanged (Fig. 6C). Consequently, COX-2 inhibitor exerts negative effects on bone marrow density and trabecular bone quality mainly through reduced bone formation, indicating that blockade of PGE<sub>2</sub> exacerbates osteopenia in Tg mice.

#### Effects of COX-2 inhibitor on mRNA expression by POBs

Because osteoblasts appeared to play a key role in osteopenia of Tg mice, the effects of celecoxib on mRNA expression of bone metabolism-related agents in POBs were analyzed using RT-PCR. Accelerated RANKL mRNA expression promoted by transgene-derived IL-1 $\alpha$  was markedly reduced in response to 0.1 mM celecoxib in Tg mice POBs, and similar inhibitory effects were observed in control POBs (Fig. 7A). Conversely, constitutively decreased expression of alkaline phosphatase mRNA in Tg mice POBs was further reduced in response to celecoxib treatment. In contrast, neither M-CSF nor GM-CSF mRNA expression displayed any response to celecoxib treatment and, importantly, GM-CSF expression remained rather high even after celecoxib treatment. According to analysis of the dose-dependent effects of celecoxib on each mRNA expression, RANKL mRNA levels were clearly decreased with increasing doses of celecoxib in Tg mouse-derived POBs, whereas GM-CSF and M-CSF remained unchanged irrespective of celecoxib dose (Fig. 7B). Interestingly, when in vitro osteogenesis by Tg mouse-derived POBs was assessed using alkaline phosphatase, von Kossa, and Alizarin red staining, the bone-forming ability of POBs dramatically decreased in response to celecoxib in a dose-dependent manner (Fig. 7, C and D).

## Discussion

As previously reported, IL-1-induced GM-CSF may play a pathogenic role in not only synovial tissue, but also bone marrow in IL-1 $\alpha$  Tg mice (11). Indeed, GM-CSF production was dramatically up-regulated at both mRNA and protein levels in synovio-cytes and POBs derived from Tg mice. GM-CSF has been reported as a strong inhibitor of osteoclast differentiation acting at the early stage (13). The inhibitory effects of GM-CSF on osteoclasts were well reflected in our FACS results of Tg mouse-derived BMCs, showing that the proportion of c-Fms<sup>+</sup>Mac-1<sup>high</sup> cells, which reportedly include abundant osteoclast precursor cells, were decreased, whereas c-Fms<sup>+</sup>Mac-1<sup>low</sup> cells comprising mature macrophages, granulocytes, and cells committed to becoming dendritic cells were increased (27, 28). These differences in phenotypic characteristics of BMCs between Tg and control mice were also identified by the observation of the increased number of F4/80<sup>+</sup> cells and CD11c cells and the decreased number of RANK<sup>+</sup> cells, which represent macrophage, dendritic cell, and osteoclast precursors, respectively (or unpublished observation).

In vitro osteoclast formation was substantially inhibited in Tg mice, and both BMCs and POBs appear to play a key role in this inhibition (Figs. 1 and 2). Inhibited formation of TRAP-positive MNCs in bone marrow culture of Tg mice (Fig. 1) and in coculture of Tg mice BMCs and control POBs (Fig. 2) was attributable to an decreased number of osteoclast precursors within the bone marrow of Tg mice as defined by FACS analysis. When POBs were Tg mouse-derived during coculture of BMCs and POBs, formation of TRAP-positive MNCs was markedly inhibited, potentially because POBs were potent producers of GM-CSF in Tg mice bone marrow, and such POB-derived GM-CSF may directly inhibit spontaneous differentiation of BMCs toward osteoclasts.

In contrast, osteogenic capacity should be considered to explain osteopenia in Tg mice. IL-1 is widely known as a catabolic agent in bone metabolism, although anabolic actions of PGE<sub>2</sub> induced by IL-1 are also widely accepted and may affect osteoblastic bone formation in Tg mice (29). Several studies have shown that PGE<sub>2</sub> stimulates new bone trabeculae and increased osteoblast numbers and activity (30, 31). In addition, systemic administration of PGE<sub>2</sub> dramatically increases bone formation and bone mass (32, 33). However, despite increased levels of PGE<sub>2</sub>, histomorphometric analysis of Tg mice revealed that new bone formation was actually abrogated, and RT-PCR analysis of Tg mouse-derived POBs indicated a decreased level of alkaline phosphatase mRNA (Fig. 3), both suggesting that the catabolic effects of IL-1 exceed anabolic effects in our Tg mice.

Regarding the therapeutic effects of COX-2 inhibitors, oral administration of celecoxib in Tg mice resulted in further deterioration of osteopenia, contrary to the expectations from recent knowledge. Inhibition of COX-2 theoretically reduces levels of PGE<sub>2</sub> and subsequent RANKL expression, leading to reduced osteoclast formation and bone resorption. However, because GM-CSF is spontaneously produced by overproduced IL-1 in Tg mice, osteoclastic bone resorption is markedly and constitutively inhibited by GM-CSF, suggesting that the inhibitory effects of celecoxib on bone resorption through reduced production of RANKL by osteoblasts may become minimized and can be neglected in Tg mice. Consequently, during celecoxib treatment, inhibition of PGE<sub>2</sub>-induced bone formation by osteoblasts may far outweigh inhibition of PGE<sub>2</sub>-induced bone resorption, resulting in further exacerbation of osteopenia. Actually, in our study, in vitro osteogenesis by Tg mouse-derived POBs was markedly inhibited in response to celecoxib (Fig. 7, C and D). Interesting results have recently suggested

that genetic ablation of COX-2 or administration of COX-2 inhibitors under normal physiological conditions reduces bone formation more than bone resorption (34, 35). In contrast, other studies dealing with collagen-induced arthritis in COX-2 knockout mice and administration of COX-2 inhibitors in several animal arthritis models have concluded that blockade of COX-2 ameliorates not only joint inflammation, but also osteopenia (19, 36). Combined with these contrasting lines of evidences, COX-2 and subsequent PGE<sub>2</sub> production may play an important role in certain pathological conditions with enhanced bone resorption, but may predominantly contribute to bone formation under normal physiological condition.

Recently, a close relationship between the immune and skeletal systems has attracted a great deal of attention due to the observation that inflammatory osteopenia is a common sequela associated with autoimmunity, and such cross-talk between the skeletal and immune systems has led to the term "osteimmunology", which is increasingly being considered as an important area (37, 38). Sato et al. (39) reported that IL-17-producing T cells (Th17) express RANKL, and the IL-23-IL-17 axis (i.e., Th17 axis) rather than the IL-12-IFN- $\gamma$  axis (i.e., Th1 axis) is critical for osteopenia in autoimmune arthritis. We believe that there is no pathogenic contribution of Th17 cells in our Tg mice due to following reasons: 1) our Tg mice represent a cytokine-driven but not immune-driven model with macrophage- and neutrophil-dominant arthritis, as reported previously (11); and 2) the arthritogenic effects of IL-17 reportedly act independent of IL-1 (40). However, a recent report suggested that IL-1 functions upstream of IL-17 to promote pathogenic Th17 cells in autoimmune encephalomyelitis (41), encouraging us to consider the pathogenic role of Th17 in our Tg mice. This issue is currently under investigation in our laboratory.

Given the paradoxical effects of COX-2 inhibitors displayed in the present results, administration of COX-2 inhibitors as a painkiller should be carefully considered, particularly in normal healthy patients. In addition, even if the patient suffers from inflammatory joint disease such as rheumatoid arthritis, indications for administration of COX-2 inhibitor should be carefully determined according to the type and stage of arthritis. For example, administration of COX-2 inhibitor may exert negative effects on osteopenia in rheumatoid arthritis patients whose arthritis is tightly controlled using a recent biological agent such as anti-TNF Ab, potentially because osteoclastic bone resorption has already been suppressed to normal levels. Taken together, we concluded that PGE<sub>2</sub> blockade may exert positive effects on excessively enhanced bone resorption observed in inflammatory bone disease, but negative effects may occur, even in the presence of inflammatory disease, if bone resorption is constitutively normal or controlled to within the normal levels. Further detailed analyses of the effects of COX-2 inhibitors on bone metabolism are necessary to provide a better understanding of the clinical benefits or risks of administration, particularly in patients with inflammatory disease.

## Disclosures

The authors have no financial conflict of interest.

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